“The aim of natural science is not simply to accept the statements of others, but to investigate the causes that are at work in nature.”

St. Albertus Magnus

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Impact of enzymatically generated peptides on the formation of alkylpyrazines in Maillard model systems and baked food products

Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD) in Applied Biological Sciences

Dutch translation of the title:

Impact van enzymatisch gegenereerde peptiden op de vorming van alkylpyrazinen in Maillard-modelsystemen en gebakken levensmiddelen

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The author,	The promoter,	The promoter,
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Gustavo Scalone

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Table of Contents

List of abbreviations.

AA	Amino acid
ALA	Alanine
ANOVA	Analysis of variance
ASN	Asparagine
a_w	Water activity
CAR	Carboxen
DVB	Divinylbenzene
FMOC	9-Fluorenylmethoxycarbonyl chloride
GC-MS	Gas chromatography coupled with mass spectrometry
GLU	Glutamic acid
GLY	Glycine
GPLC	Gel permeation liquid chromatography
HPLC	High performance liquid chromatography
HS	Headspace
LC-MS	Liquid chromatography coupled with mass spectrometry
LEU	Leucine
LYS	Lysine
MALDI – TOF – MS	Matrix-assisted laser desorption/ionization – time of flight - mass spectrometry
MALDI – TOF – Tandem MS	Matrix-assisted laser desorption/ionization – time of flight - tandem mass spectrometry
OPA	Ortho-phthaldialdehyde

Pep	Pepsin
Proteinase *Asp*	Proteinase from *Aspergillus melleus*
PDMS	Polydimethylsiloxane
SER	Serine
SIM	Selective ion monitoring
SPME	Solid phase microextraction
SWOT (Analysis)	Strengths, Weaknesses, Opportunities and Threats

INTRODUCTION AND AIM OF THE THESIS

Food product's perception and appreciation is given generally by a combination of factors such as appearance, taste, flavor, composition and nutritional value. Flavor, among the aforementioned factors, is defined as the sensory impression of food and is primarily determined by the chemical senses of taste and smell. Aroma is defined as the smell associated with a food product, and it is the main determinant of food flavor. While there are only five universally recognized basic tastes such as sweet, sour, bitter, salty, and umami, the number of food aromas is enormous. The appreciation of aromas can lead to the ultimate acceptance of a food product as for example it can stimulate the desire of consumption of a person. Meanwhile, unpleasant aromas might indicate or be generally associated with spoilage. A food product's flavor, therefore, can be easily altered by changing its aroma while keeping its taste similar. Therefore, it is understandable that the control and optimization of flavor are the ultimate challenge for the food and flavor industry. The reactions that occur between the different ingredients of a food product during cooking, baking, and preservation of all kinds are of a great importance for the production of aroma, taste and color. Among these reactions, the Maillard reaction possesses a remarkable importance.

The Maillard reaction is the major route for thermal generation of flavor, initiated by reducing sugars and amino compounds. It is a very complex cascade of interdependent chemical reactions which has been the topic of many research papers since it was first identified by Louis Camille Maillard in 1912,[1] further elucidated by Hodge in 1953[2] and later described in detail by Ledl in 1990.[3] The Maillard reaction comprises a set of complex chemical reactions, which are initiated when a free amino group of an amino acid, a peptide, a protein or an amine reacts with the carbonyl group of a reducing sugar. This non-enzymatic browning reaction gives rise to modifications in color, aroma, taste and nutritional value of thermally treated foods. However, it might produce undesirable compounds as well, such as furan that is toxic.

The aromas in most thermally processed foods, such as bread, cereal products, roasted peanuts, and roasted coffee, are largely due to the Maillard reaction. Currently, more than 2500 different flavor compounds have been identified.[4-6] Among these Maillard–type flavors, heterocyclic compounds with desirable aromas and low odor thresholds make the most significant contribution.[5] Substituted pyrazines and pyrazine itself are specific Maillard reaction products with low odor threshold values, and are known to contribute significantly to the unique roasted, nutty, meaty, earthy, popcorn-like aroma of many heated food products.[5,7]

The reaction between free amino acids and carbonyl compounds has been studied extensively,[8] whereas the Maillard reaction between peptides and proteins with carbonyl compounds has been less investigated.[9-11] In general, food products are known to contain small

amounts of free amino acids except for some particular food products like salt fermented shrimp paste [12] and fish sauce [13], whereas there are several peptide containing food products which have been reported to develop flavor and color formation due to the Maillard reaction [14-20]. Oligopeptides have been recognized as important flavor enhancers and precursors of the Maillard reaction and it has been shown before that peptides may generate pyrazines.[9,21-23] Despite these facts, their potential contribution to the overall pyrazine formation in food has been studied less.

A limited number of studies have investigated the generation of volatiles in protein – carbohydrate and peptide – carbohydrate models. Nevertheless, these studies were evaluated under experimental conditions that are hardly comparable to those reflecting the composition or manufacturing processes of food products in general.[24-30] The challenge of creating model systems to evaluate the formation of flavor compounds, is to make them reflect real food systems, therefore providing useful information to the food industry.[31]

The aim of this thesis is to study the formation of pyrazines in different Maillard model systems containing peptides, proteins, amino acids and glucose in a variety of conditions which can be related and applied to the food industry. Moreover, the work was planned in order to achieve the following objectives.

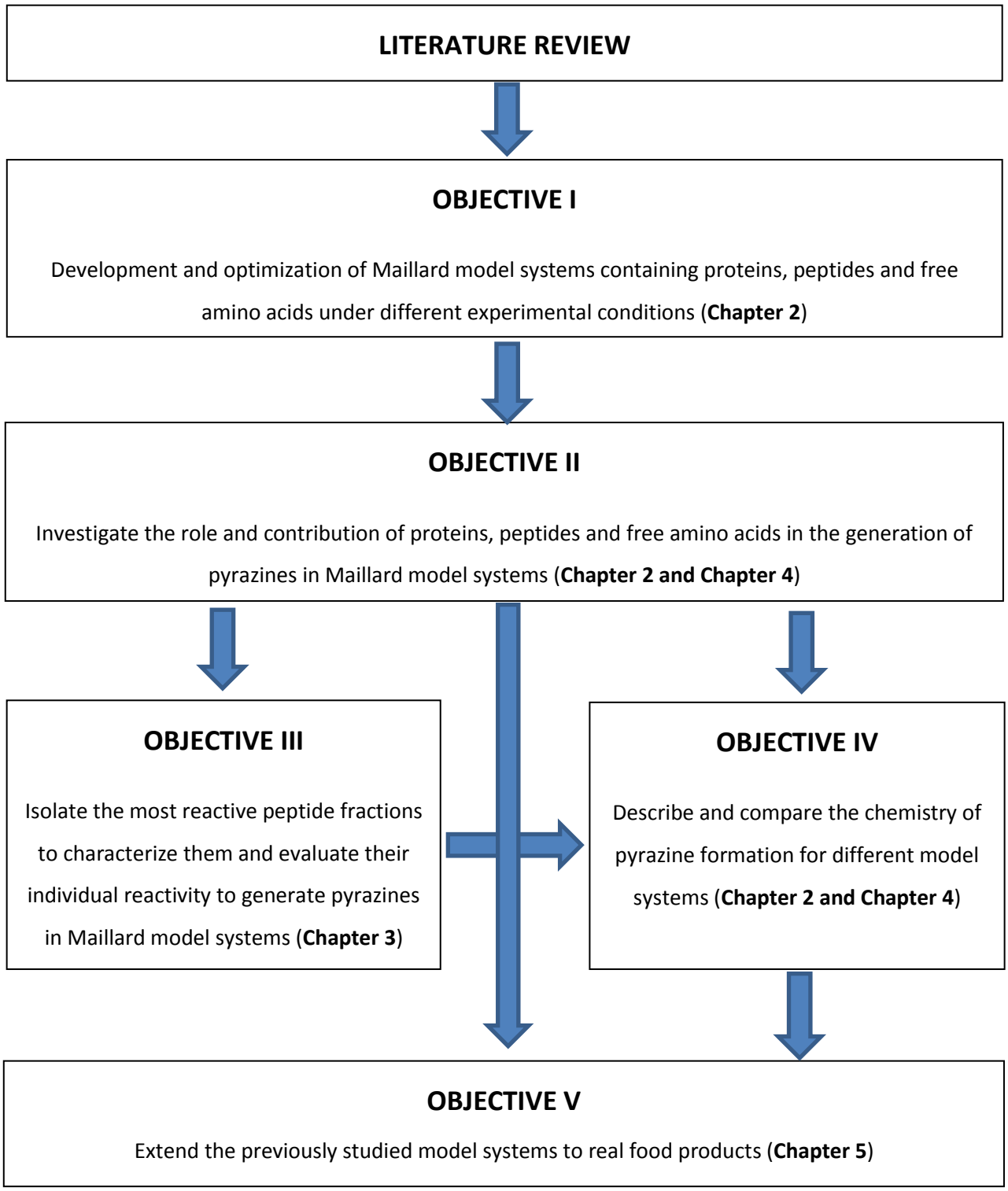
The first objective was to develop more complex Maillard model systems which contain proteins, peptides and amino acids, under different experimental conditions. The Maillard model systems were made using glucose, whey protein isolate and tryptic hydrolyzed whey protein as a source of peptides and amino acids (**Chapter 2**). The model system was further optimized in order to obtain repeatable and reproducible data.

The second objective was to elucidate the role and overall contribution of proteins, peptides, amino acids (and glucose) on the generation of pyrazines in the aforementioned model systems. Six different whey protein hydrolysates, and a native whey protein isolate were used and compared in Maillard model systems containing glucose (**Chapter 2 and Chapter 4**).

The third objective was to isolate the different peptides contained in one of the hydrolysates, with the objective of using them in Maillard model systems. Tryptic hydrolyzed whey protein was separated into six fractions of different molecular weight using GPLC. The resulting fractions were identified using MALDI-TOF-MS and MALDI-TOF-Tandem MS (**Chapter 3**). Moreover, the fractions were used to make Maillard model systems with glucose to evaluate their pyrazine formation (**Chapter 3**).

The fourth objective was to investigate, describe and compare the chemistry of pyrazine formation in the model systems which generated the highest amounts of pyrazines. Further, simplified models systems were made by replacing glucose with two dicarbonyl substances with the objective to elucidate the different formation mechanism of the generated pyrazines (**Chapter 4**).

The fifth and last objective was to extend some of the previous model systems to a real food product. Two different baked products were made using whey protein hydrolysates among the ingredients (**Chapter 5**). Additionally, their ability to generate specific aromas was evaluated using GC-MS, and finally their acceptance was evaluated by a sensory panel.



CHAPTER 1

Literature review. Peptides and their importance in food, the Maillard reaction and pyrazine formation in Maillard model systems

LITERATURE REVIEW

1.1 Peptides, definition and general aspects

Peptides (**Figure 1**) are amino acid polymers in which the carboxyl group of one amino acid binds with the amino group of the second amino acid via an amide bond (peptide bond). The combination (condensation) of two amino acids yields a dipeptide, three molecules of amino acids produce a tripeptide and so on. The formation of a dipeptide from two amino acids is accompanied by the loss of a water molecule. Each linear peptide contains one amino acid with a free carboxyl group, which is the *C*-terminal amino acid, and one amino acids with a free amino group, the *N*-terminal amino acid. In addition to linear peptides, the condensation of amino acids may also lead to cyclic structures, which have no free carboxyl group and no free amino group.[32]

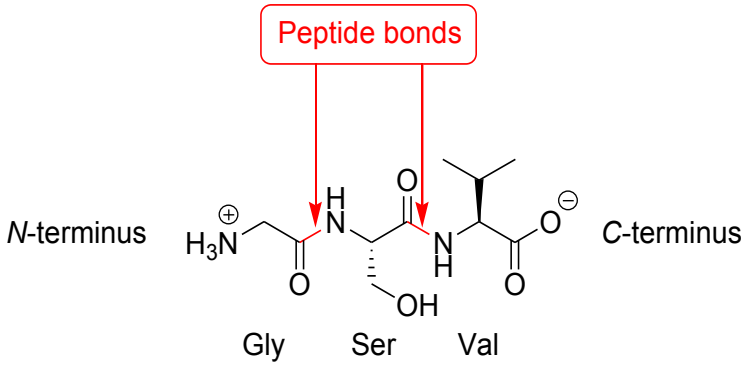


Figure 1. Chemical structure of the peptide bonds of a tripeptide.

Plants and microorganisms are able to generate all 21 amino acids, 20 of which are encoded for protein synthesis (**Appendix 1**), while selenocysteine is encoded in a special way called translational recoding.[33] Nevertheless, plants and microorganism can additionally synthesize many more amino acids. Food contains numerous other less common amino acids in addition to those that are constituents of proteins (proteinogenic) and modified proteinogenic amino acids. It is estimated that there are around 700 amino acids known in nature, of which at least 300 are found in plants.[32] In biochemistry, these non-protein amino acids are often classified as secondary metabolites, as they are the product of three major routes: modification of an existing (often proteinogenic) amino acid, modification of an existing pathway and by novel pathways. These amino acids become the biosynthetic precursors of many biologically active nitrogenous compounds, such as signaling molecules, vitamins, alkaloids, bile acids and pigments. Additionally some non-protein amino acids are often bound in peptides. In living organisms, peptides arise either from amino acids by simple

biosynthesis (in contrast to proteins, this is without the use of the proteosynthetic apparatus), or by hydrolysis of precursors produced in protein synthesis (with possible chemical modification).[32]

Peptides are classified with Greek prefixes as di-, tri-, tetra-, penta-, decapeptides, etc., according to the number of amino acid residues incorporated. In longer peptides, the Greek prefix may be replaced by Arabic figures. Formerly, peptides containing fewer than 10 amino acid residues were classified as oligopeptides (Greek oligos = few) and peptides with 10–100 amino acid residues were called polypeptides.

From a chemical point of view, a differentiation between polypeptides and proteins is ambiguous. According to the currently accepted nomenclature rules, “oligopeptides” are composed of fewer than 15 amino acids, “polypeptides” contain approximately 15–50 amino acid residues, and the expression “protein” is used for derivatives containing more than 50 amino acids.[34]

The nomenclature formally considers peptides as N-acyl amino acids. Only the amino acid residue at the carboxyl terminus of the peptide chain keeps the original name without suffix, while all others are used with the original name and the suffix -yl.[34]

A further simplification of a peptide formula is achieved by using either the three-letter code, or the one letter code for amino acids (see **Appendix 1**). Linear peptide sequences usually are written, starting with the amino terminus on the left side and the carboxyl terminus on the right side. When nothing is shown attached to either side of the three-letter symbol, it should be understood that the amino group (always on the left) and carboxyl group, respectively, are unmodified. The sequence of amino acids that are connected to a peptide or a protein are called the “primary structure”. If the sequence of a peptide is completely known, the three-letter code symbols are listed sequentially, divided by a hyphen “–”, which symbolizes the peptide bond,[34] while for long sequences as in proteins the one letter code is often preferred.

Peptide research has experienced considerable development over the course of the past few decades. The progress in this important discipline of natural product chemistry is reflected in a flood of scientific data. Peptide chemistry contributes considerably to the research in the life science area. Therefore, it is understandable that this science it is becoming an attractive research discipline.[34]

A number of very important physiological and biochemical functions of life are influenced by peptides. Peptides are involved as neurotransmitters, neuromodulators and hormones in receptor-mediated signal transduction. More than 100 peptides with functions in the central and peripheral nervous systems, in immunological processes, in the cardiovascular system and in the intestine are known. Peptides influence cell-cell communication upon interaction with receptors and are involved in a number of biochemical processes.[34] Therefore, several studies regarding the biological activity of peptides describe a considerable number of activities such as antioxidant,[35-39] antimicrobial,[35-36,40-41] antihypertensive,[35-37,42] anti-thrombotic,[35,37] hypocholesterolemic and

hypotriglyceridemic,[37] antiobesity,[37,43] opioid agonist and antagonist,[35-36,40] inmunomodulating,[24,35,40] cytomodulating,[35] cytotoxic[44] and allergenic properties[40,44]. Some peptides are multifunctional and can exert more than one of the effects mentioned.

1.2 Importance of peptides in food

Peptides are found in a large variety of food products. They can be generated via autodigestion of proteins, as in dry cured ham[45-46] and meat[47], or via fermentation in for instance the sourdough of bakery products, as well as in the fermented products of various fish, shrimp, cocoa and beer.[12-13,20,48-51] Peptides can also be obtained via the deliberate addition of particular enzyme preparations (**Table 1**) or starting cultures, which promote protein hydrolysis. Dairy products, such as kefir, yogurt and various types of cheese, are for instance obtained via this method.[17,19]

With increasing length, peptides have a greater tendency to exhibit elements of secondary structure and therefore possess various functional properties similar to proteins. These include solubilizing, emulsifying, gelling and foaming properties.[40]

Several authors agree that hydrolyzed proteins and therefore peptides are useful and important as flavoring agents[52-53] and are responsible for production of bitter, sweet, sour, salty and umami flavors.[21,40,45-46,54-56] Additionally, they have an important role in functional foods as mentioned in **Section 1.1**.

1.3 Protein hydrolysis

As mentioned before, peptides can occur in food products as they are generated for instance by hydrolysis of proteins via autodigestion, fermentation or addition of enzymes. In several cheese types, protein hydrolysis is promoted by adding bacterial or fungal cultures that produce a variety of proteolytic enzymes. In other cases, protein hydrolysis is achieved by direct addition of specific proteolytic enzymes (see **Table 1**).

Enzymatic hydrolysis seems to be the most promising way to obtain different peptides, this due to their specificity of cleavage, which makes it easier to control than chemical hydrolysis.

Enzymes are specific in action and bring substrates together in favorable orientations to promote the formation of the enzyme substrate complex (transitory state). The properties and spatial arrangements of amino acids residues that form the active site of an enzyme are responsible for its specificity, and determine which molecules can bind and be a substrate for these enzymes.[7] Generally an enzyme's name is often derived from its substrate or the chemical reaction it catalyzes, with the word ending in –"ase" as in peptidase, lipase, alcohol dehydrogenase and DNA polymerase.

Peptidases, are a group of enzymes that perform proteolysis (breakdown of proteins into smaller polypeptides or amino acids). Peptidases are classified by several factors, including their optimal pH activity and proteolytic mechanisms. For instance, peptidases are classified as alkaline, neutral or acid. Further, it is possible to classify peptidases into two groups, exopeptidases and endopeptidases. Exopeptidases are peptidases that catalyze the cleavage of the terminal (or the penultimate) peptide bond; this cleavage releases a single amino acid or dipeptide from the peptide chain. Exopeptidases are subdivided in aminopeptidases and carboxypeptidases when the cleavage occurs at the *N*-terminal or *C*-terminal amino acid, respectively.[57] Endopeptidases are peptidases that cleave peptide bonds of nonterminal amino acids. Endopeptidases are broadly used in the food industry (**Table 1**) and their further sub-classification is normally given based on their specificity of cleavage. Some examples are provided in **Table 2**.

Table 1. Peptidases authorized by the European Union to be used in food products.

Regulation (EC) No 1332/2008 of the European Parliament and of the Council of 16 December 2008 on food enzymes and amending Council Directive 83/417/EEC, Council Regulation (EC) No 1493/1999, Directive 2000/13/EC, Council Directive 2001/112/EC and Regulation (EC) No 258/97 (Text with EEA relevance)			
Reference Number	Food enzyme	Source	Food uses
2013/07	Thrombin	Cattle or pig blood	Meat and fish products
2013/09	Food enzyme consisting of trypsin and chymotrypsin	Pig pancreas	Dairy processing
2013/27	Chymotrypsin	*Bacillus licheniformis*	Protein hydrolysis
2014/35	Mucorpepsin	*Rhizomucor miehei*	Dairy processing and beverage processing
2014/55	Food enzyme consisting of trypsin, chymotrypsin, elastase and carboxypeptidase	Porcine pancreatic glands	Dairy processing intended for infant formulae and food for special medical purposes.
2015/05	Chymosin	*Kluyveromyces lactis* (strain CIN)	Dairy processing
2015/16	Chymosin	*Kluyveromyces lactis* (strain CHY)	Dairy processing
2015/36	Trypsin	Porcine pancreatic glands	Protein hydrolysis
2015/40	Food enzyme consisting of trypsin and chymotrypsin	Porcine pancreatic glands	Dairy processing
2015/45	Papain	*Carica papaya* (papaya)	Dairy processing, egg processing, production of bakery and cereal-based products

2015/52	Carboxypeptidase C	*Aspergillus niger* (strain PEG)	Dairy and meat processing, protein hydrolysis, flavoring production
2015/53	Peptidase	Edible pumpkin, squash or gourd	Dairy processing
2015/54	Peptidase	Edible *Galium verum*	Dairy processing
2015/55	Peptidase	Edible ginger roots	Dairy processing
2015/56	Peptidase	Edible papaya	Dairy processing
2015/57	Peptidase	Edible parts of *Cynara* family (thistle, artichoke)	Dairy processing
2015/58	Peptidase	Edible ectomycorrhizal mushrooms (truffles, porcini, morel)	Dairy processing
2015/59	Peptidase	Edible pinapple	Dairy processing
2015/60	Peptidase	Edible kiwi	Dairy processing
2015/61	Peptidase	Edible fig	Dairy processing
2015/76	Food enzyme consisting of peptidase, leucyl aminopeptidase, oryzin andaspergillopepsin I	*Aspergillus oryzae*	Production of bakery products, dairy processing, flavoring production, production of cereal-based distilled alcoholic beverages, protein hydrolysis, yeast processing
2015/80	Food enzyme consisting of aspergillopepsin I and II	*Aspergillus niger var. macrosporus* (strain DBD-0406)	Production of wine
2015/87	Thermolysin	*Geobacillus caldoproteolyticus* (strain DP-Fzj32)	Protein hydrolysis
2015/100	Chymosin	*Aspergillus niger var. awamori* (strain DSM 29544)	Dairy processing
2015/101	Chymosin	*Aspergillus niger var. awamori* (strain DSM 29545)	Dairy processing
2015/102	Chymosin	*Aspergillus niger var. awamori* (strain DSM 29546)	Dairy processing
2015/103	Mucorpepsin	*Rhizomucor miehei* (strain DSM 29547)	Dairy processing
2015/122	Mucorpepsin	*Rhizomucor miehei*	Dairy processing
2015/130	Thermolysin	*Geobacillus stearothermophilus* (strain AE-TP)	Dairy, egg, meat, yeast and fish processing; flavoring production, protein hydrolysis

2015/152	Membrane alanyl aminopeptidase	*Lactococcus lactis*	Dairy processing
2015/167	Ficin	*Ficus glabrata*	Yeast, egg, soyam fish and meat processing; beer and other cereal-based beverages
2015/168	Bromelain	*Ananas comosus*	Yeast, egg, soyam fish and meat processing; beer and other cereal-based beverages
2015/214	Pancreatin	Porcine pancreas and duodenum	Production of infant formula and food for special medical purposes

Table 2. Types and families of endopeptidases, adapted from Barret and Rawlings[58]

Catalytic type	Family (representative member, other members)
Serine endopeptidases	Chymotrypsin, trypsin, pancreatic elastase, peptidase E, chymase, trypase, cathepsin G, plasmin, batroxobin, flavoxobin, hypodermin C
Cysteine endopeptidases	Papain, actinidin, chymopapain, papaya endopeptidases III and IV, ficin, calotropin, bromelains, cathepsins B, H, L, S
Aspartic endopeptidases	Pepsin, gastricsin, chymosin, renin, cathepsins D and E, penicillopepsin, Human immunodeficiency virus (HIV) endopeptidase, Thermopsin
Metallo-endopeptidases	Thermolysin, bacillolysin, snake venom metallo-endopeptidase, astacin, insulinase, mitocondrial processing peptidases, stromelysin, matrin
Unknown	Bacterial leader peptidase I, eukaryote leader peptidases

1.4 The Maillard reaction, the most important reaction in food chemistry

For as long as food has been cooked, baked, roasted or fried, the Maillard reaction has played an important role in the development of a unique aroma and an agreeable brown color. The Maillard reaction is the major route for thermal generation of flavor, initiated by the condensation reaction between a free amino group of an amino acid, peptide or protein and a carbonyl group of a reducing carbohydrate. Although the Maillard reaction seems to be simple, it is a complex cascade of interdependent chemical reactions (**Figure 2**) which has been the topic of many research papers since it was first identified by Louis Camille Maillard in 1912[1], further elucidated by Hodge in 1953[2] and later described in detail by Ledl in 1990.[3] The Maillard reaction is quite complex. Therefore, in order to understand it, it is necessary to subdivide it. Hodge et al. subdivided the Maillard reaction as follows.

I) Initial stage: the resulting products are colorless, without a strong absorption in the ultraviolet region (<280 nm). This stage involves the sugar-amine condensation and the Amadori or Heyns rearrangement.

II) Intermediate stage: products are colorless or slightly yellow, with a strong absorption in the ultraviolet. This stage involves sugar dehydration, sugar fragmentation and amino acid degradation (Strecker degradation).

III) Final stage: the obtained products are highly coloured and called melanoidins to distinguish them from melanins produced by enzymatic browning. This final stage comprises aldol condensation, aldehyde-amine condensation and formation of heterocyclic nitrogen compounds.

It is well-known that the Maillard reaction participates in the formation of aroma during cooking and thermal processing in the food industry. The aromas of roasted meat, steamed fish, boiled eggs, coffee, roasted cocoa and nuts, chocolate, malt, popcorn, bread, beer, whisky and many others, owe their flavor largely to the Maillard reaction.[4,59-62] However, some products of the Maillard reaction are studied due to their known or potential toxicity. Furan and acrylamide are well-known food toxicants which can be generated due to the Maillard reaction.[63-67]

Control and optimization of the generation of flavor and toxic compounds are extremely important factors for the food and flavor industry. For this reason, research groups worldwide dedicate themselves to understanding and elucidating the chemistry involved in this important reaction in foods and several reviews have been published in the literature.[6,31,61-62,66,68-70]

The different stages of the Maillard reaction are responsible of the generation of several compounds, for such reason, the initial, intermediate and final stages of this reaction will be described in detail in the coming sections.

1.4.1 Initial stage of the Maillard reaction

The Maillard reaction is initiated by the reaction of a carbohydrate **1** and a nitrogen source, unprotonated amine group (amino acid, peptide, or protein) to form N substituted N-glycosylmine **5** or **12** with the loss of a water molecule via aldimine **4** (**Scheme 1**), or a ketimine **11** (**Scheme 2**). An aldose **2** gives rise to an aldimine **4** and a ketose **10** gives a ketimine **11**. The glycosylimines are unstable and undergo an intramolecular arrangement called the Amadori rearrangement in the case of aldimine **4**, and the Heyns rearrangement in the case of ketimine **11**. These transformations

produce the Amadori products 1-amino-1-deoxy-2-ketose **8** and Heyns products 2-amino-2-deoxyaldose **15** and 2-amino-2-deoxy-3-ketose **17**. Still, no color is produced at this stage of the reaction.

It is known that the major loss of lysine in food proteins is primarily attributed to the Maillard reaction (**Table 3**), and specifically due to the Amadori and Heyns rearrangement, but other amino acid residues such as arginine, asparagine, and glutamine are also capable of reacting with reducing sugars.[34]

In further stages of the Maillard reaction, carbohydrate fragmentation, condensation, elimination, dehydration and cyclization reactions occur simultaneously, resulting in diverse reaction products.[3,5]

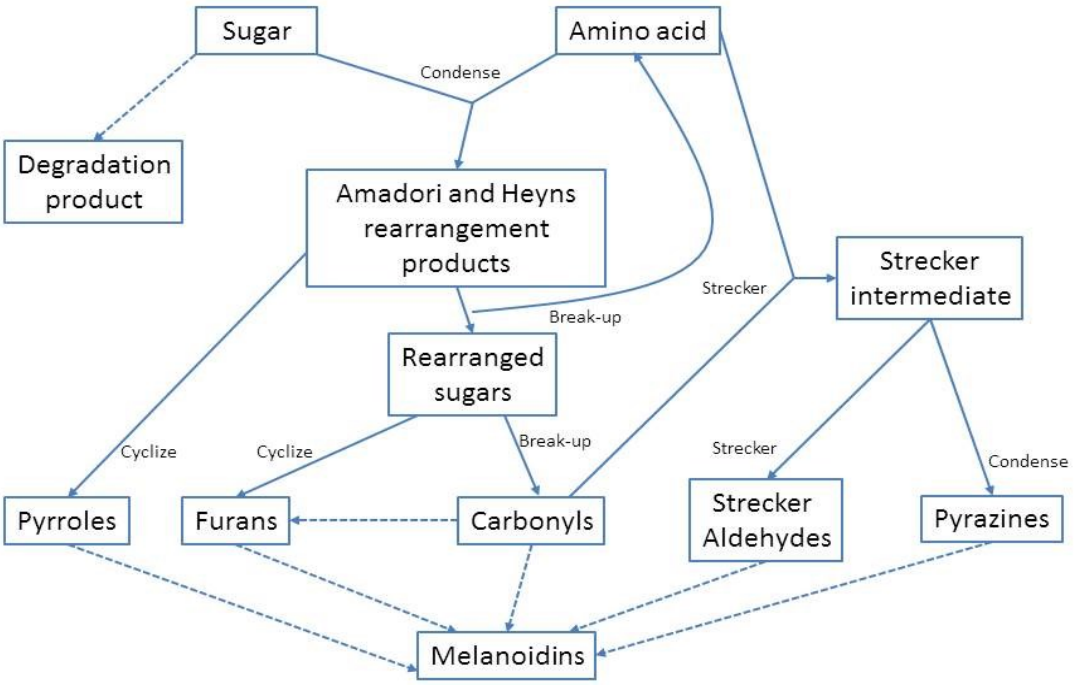
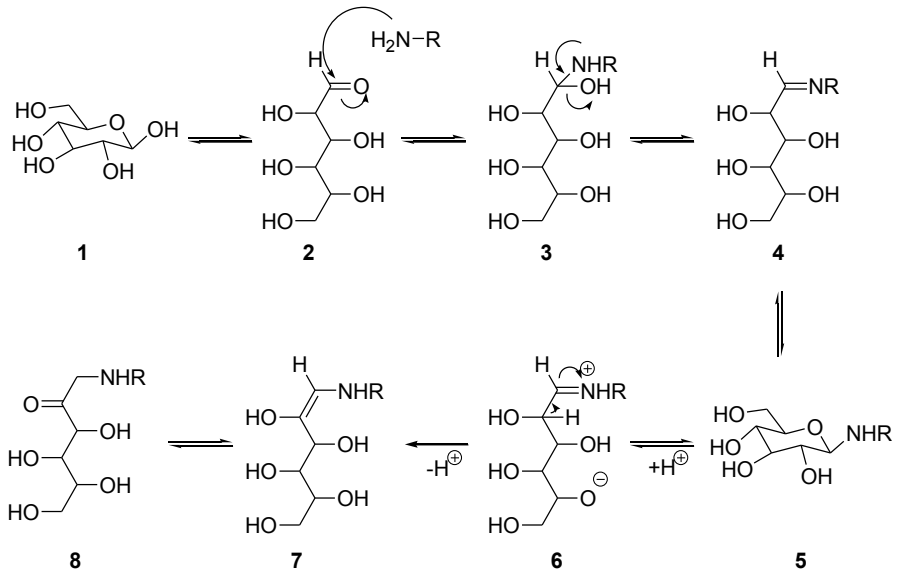
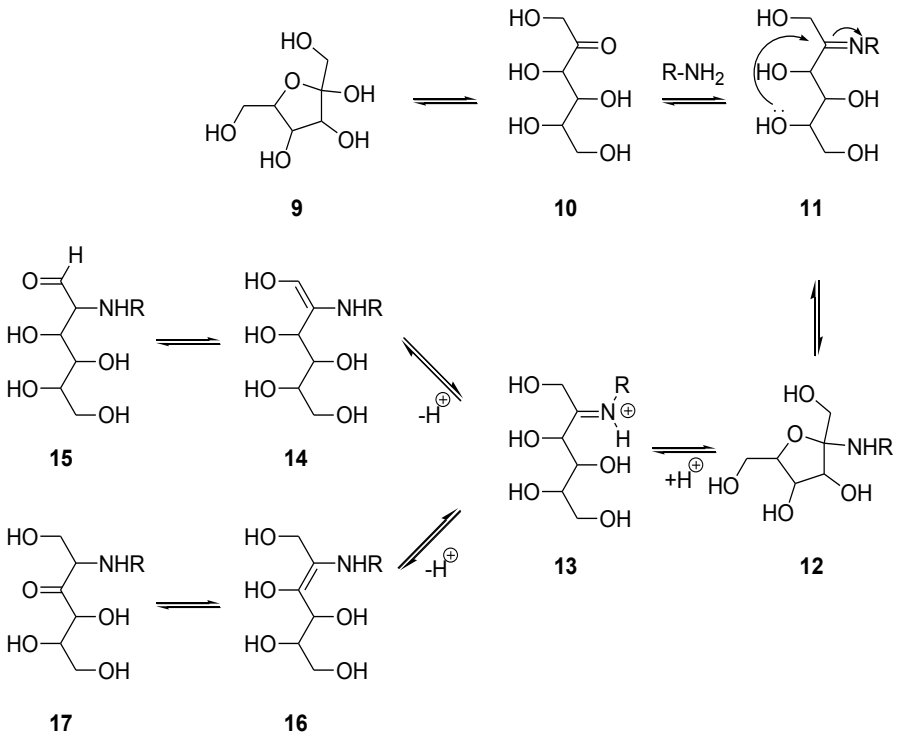


Figure 2. Simplified scheme of flavor generation by the Maillard reaction, adapted from Jousse[6]



Scheme 1. Early stage of the Maillard reaction: sugar amine condensation to form the N-substituted glycosylamine **5**, followed by the Amadori rearrangement, adapted from Ledl[3]



Scheme 2. Early stage of the Maillard reaction: sugar amine condensation to form the N-substituted ketosylamine **11**, followed by the Heyns rearrangement

Table 3. Nutritional consequences of the Maillard reaction in milk products, adapted from Mauron[71]

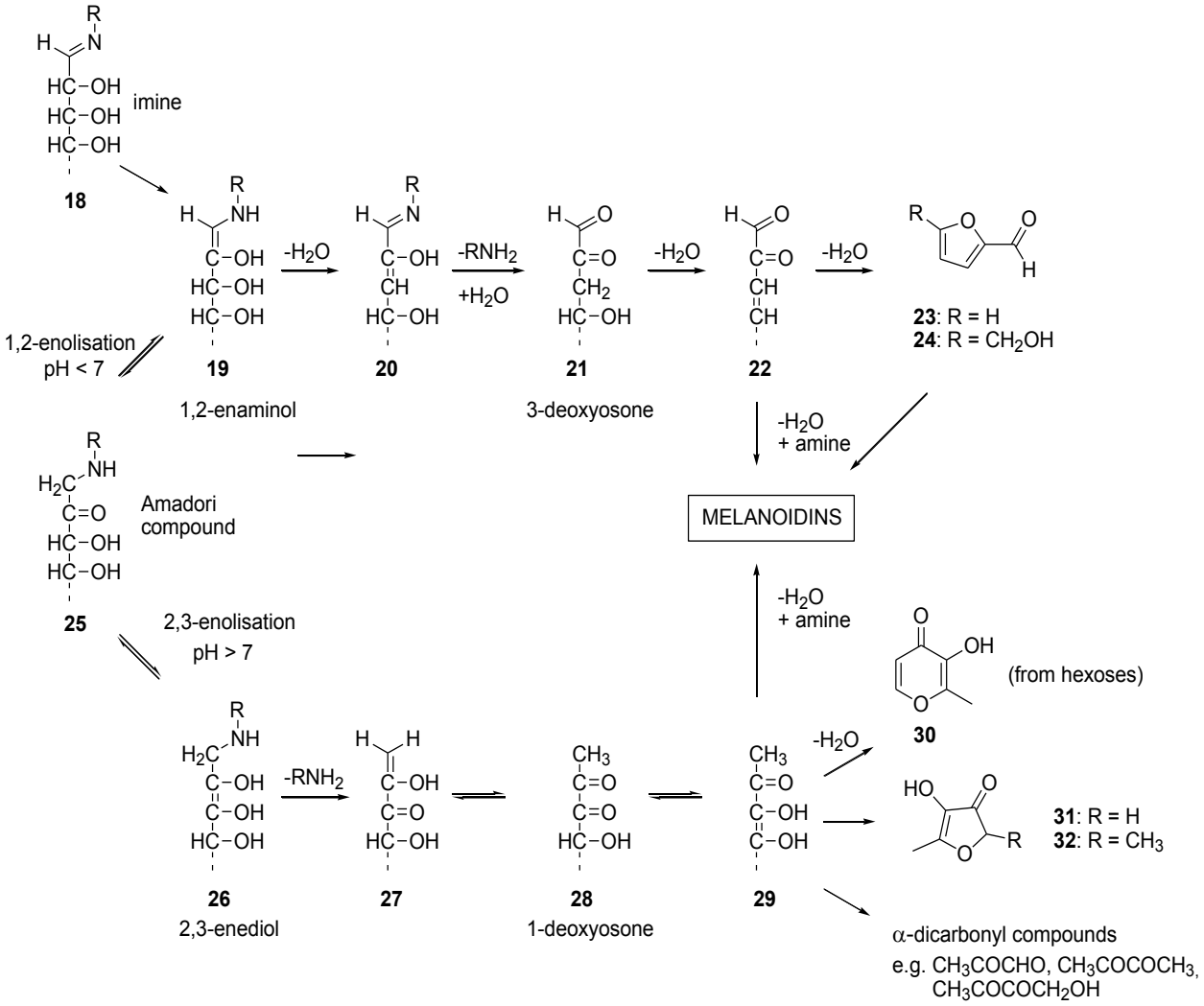
Product	% blocked lysine
Raw milk	0.0
Freeze-dried milk	0.0
Pasteurized milk 74 °C 40 sec	0.0
UHT pasteurized milk 140 °C 3 sec	0.0 – 2.0
Spray dried milk	0.0 – 3.0
Sweetened condensed milk	8.0 – 12.0
Roller dried (no preconcentration)	10.0 – 15.0
Evaporated milk	15.0 – 20.0
Drum dried milk	20.0 – 30.0

Several aspects of the reactivity of peptides and carbohydrates in the early phase of the Maillard reaction have been studied. For instance, monosaccharides have been found to be more reactive than disaccharides.[72] In addition, glucose was found to be more reactive with peptides when compared to fructose in the formation of the Amadori or Heyns products respectively.[73-74]

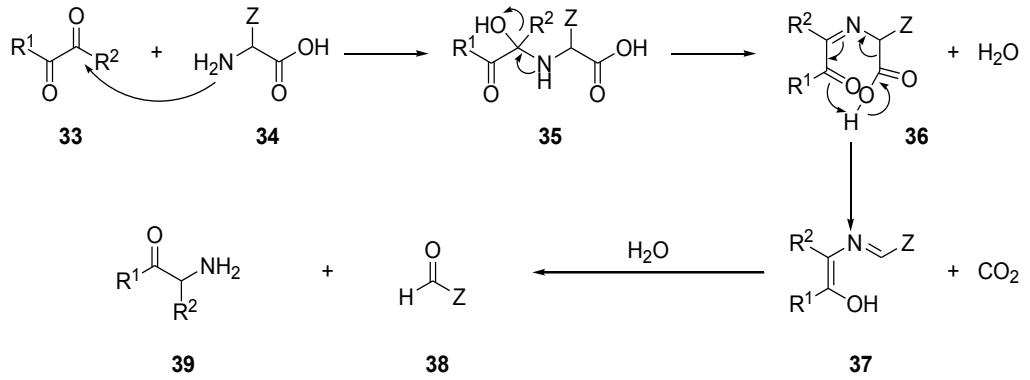
The reactivity of peptides to form Amadori and Heyns products was evaluated also according to their sequence. There exist several publications based on model systems, however, Van Lancker et al.[9] concluded that it is not easy to evaluate this reactivity. This is due to the fact that, under some conditions such as low pH or high temperature, the resulting ketosylamine/aldosylamine participates rapidly in further rearrangement and condensation reactions of the Maillard reaction.

1.4.2 Intermediate stage

The intermediate stage begins with the degradation of the Amadori and Heyns rearrangement products. The reaction can take place spontaneously even at ambient or room temperature[8], and proceeds in numerous transformations involving various pathways i.e. dehydration, cleavage (**Scheme 3**) and Strecker degradation (**Scheme 4**).



Scheme 3. Intermediate stage of the Maillard reaction adapted from Nursten[8]



Scheme 4. Strecker degradation pathway, adapted from Adams and De Kimpe [5]

1.4.2.1 Dehydration and cleavage pathway

The degradation of the Amadori rearrangement product **25** is dependent on the pH of the system (**Scheme 3**). At pH 7 or below, it undergoes mainly 1,2-enolization with the formation of furfural **23** (when pentoses are involved) or hydroxymethylfurfural **24** (when hexoses are involved). Pyrroles and furfural can be derived from the 1,2-enolization pathway. The dehydration of an Amadori compound followed by cyclization lead to the formation of pyrroles. At pH > 7, the degradation of the Amadori compound **25** is thought to involve mainly 2,3-enolization, where reductones, such as 4-hydroxy-5-methyl-2,3-dihydrofuran-3-one **31** are formed. Retroaldolization and oxidative cleavage of reductones leads to flavor compounds and precursors, such as acetaldehyde, methylglyoxal, butane-2,3-dione, and acetic acid. All of these compounds are highly reactive and take part in further reactions.

1.4.2.2 Strecker degradation pathway

The Strecker degradation pathway (**Scheme 4**) involves the degradation of α-amino acids initiated by dicarbonyl compounds. It involves the oxidative deamination and decarboxylation of an amino acid **34** in the presence of a dicarbonyl compound **33**. This leads to the formation of Strecker aldehydes **38**, containing one less carbon atom than the original amino acid, and an α-aminoketone **39**.[5]

The Strecker aldehyde **38** can take part in aldol condensation leading to nitrogen-free polymers or it can react with amino compounds to give melanoidins via aldimines. Additionally, since Strecker aldehydes **38** are volatile, they often contribute to the flavor of several food products. Further, the formation of α-aminoketones **39** also participates in the formation of aroma compounds. Two molecules of this compound can condense to form a dihydropyrazine. The formation of pyrazines from the dihydropyrazine involves a final oxidation step, with the exception of the cases when an amino acid with an α-hydroxy group (threonine, serine) is present in the sidechain of the dihydropyrazine.[75]

The previously formed α-aminoketones **39** not only generate pyrazines, they are also precursor of pyrroles, imidazoles and oxazoles under pyrolysis conditions.[76-77] The intermediate α-aminoketones **39** can react with any aldehyde in the reaction mixture to form an imine which in turn can either cyclize to form oxazoles or react with an amino compound and then cyclize to form an imidazole after an oxidation step. The formation of pyrroles occurs through aldol condensation of acetaldehyde with different α-aminoketones **39** followed by cyclization and loss of water.[77]

1.4.2.3 Other mechanisms occurring during intermediate stages of the Maillard reaction

Cysteine and methionine can also undergo Strecker degradation and transamination. However, the sulfur-containing heterocycles are formed by the direct or indirect reaction of H_2S released from the sulfur-containing amino acids with previously formed compounds such as dicarbonyl compounds, aldehydes, furan and other heterocyclic compounds.[78] Consequently, there are several formation pathways for the generation of sulfur-containing heterocycles.[8]

Proline and hydroxyproline are not primary amines; therefore, they cannot undergo Strecker degradation, nevertheless, they form specific volatiles. They can participate in reactions in which they preserve their pyrrolidine ring as is the case of 2-acetyl-1-pyrroline, a well-known powerful and characteristic flavor compound.[5] Additionally, both amino acids can expand the pyrrolidine ring forming 2-acetyltetrahydropyridine, which is the typical bread flavor compound. Several other proline and hydroxyproline specific volatiles and their formation mechanisms have been reported.[5,79-81]

1.4.3 Final stage

This stage involves condensation of the sugar degradation compounds formed in the intermediate stages with each other or with amino compounds to form aldimines and ketimines, and finally lead to brown nitrogenous polymers and copolymers, called melanoidins.[69] The chemistry of these compounds is not well-known and their formation mechanism also remains unclear. The molecular weight of these compounds increases as browning proceeds.

1.5 The Maillard reaction as a pathway for the formation of flavor and aroma compounds

The Maillard reaction produces hundreds of volatile compounds, among which many contain heterocyclic systems.[3] The Maillard reaction has been studied extensively in model systems between amino acids and reducing sugars. Elmore and Mottram compiled a list of volatile compounds that had been identified in Maillard model systems from 38 papers published between 1985 and 2002.[68] Fifteen amino acids and seven sugars yielded 621 volatile compounds. This demonstrates how a large number of compounds can contribute to aroma.[68,82] Some of the flavor compounds like alcohols, ketones and aldehydes are generally produced in high quantities due to the Maillard reaction in food products, while others, such as sulfur-containing heterocyles are generated in trace levels.[59] However, these yields of production are not necessarily related to their overall

contribution to the flavor perception in a determined food product, which is due to differences in their odor detection thresholds. The odor detection threshold is defined as the lowest concentration of a certain odor compound that is perceivable by the human sense of smell. Maltol (3-hydroxy-2-methyl-4*H*-pyran-4-one) in water for example has an odor threshold value of 9.0 mg/kg, while for 2-acetyl-1-pyrroline the value is 0.1 µg/kg. The odor threshold value of 2-ethyl-3,5-dimethylpyrazine in water is 0.04 µg/kg.[83] At the same time, it is possible to evaluate the importance of a flavor compound in a determined food product by calculating its aroma value. The aroma value of a substance is defined as the ratio between the concentration of a determined compound over its threshold value. Therefore, in general, a flavor compound contributes to the overall aroma of a food whenever it is present in a concentration higher than its threshold value. Of all the volatile compounds, only a limited number are important for the aroma of a food. Substances that possesses high aroma values, and in consequence provides characteristic aromas to a determined food are called key odorants.[83]

Recently, Dunkel et al. presented a review regarding the human perception of aroma; the authors concluded that from about 10000 volatiles expected in food products only 3% are considered key odorants.[82] Compounds like oxygen-, nitrogen- or sulfur-containing heterocycles can determine the flavor of a food even when they are generally present at trace levels, which is due to their low threshold values.[59]

In order to understand how so many different flavor compounds can be formed during the Maillard reaction, it is necessary to mention some of the factors that affect it. The Maillard reaction is highly dependent on factors such as water activity (a_w), time, temperature and the pH of the system. Some of these factors, time and temperature, can affect the overall Maillard reaction, while the effect of a_w and pH can be manipulated to promote or hinder the formation of specific products.

Temperature is highly related with the production of Maillard products, where the rate of the overall reaction and production of melanoidins increases 3 – 6 times with a 10 ^oC rise in temperature. For this reason, refrigeration is an effective method to control the Maillard reaction. Moreover, it is known that most foods will not exhibit any browning below -10 ^oC.[8]

The Maillard reaction can be controlled to some extent by modifying the values of a_w, but the effect is not straightforward. At high a_w values the reaction is hindered by dilution of the reagents. On the contrary, at low a_w values, the concentrations of reactants increase, but they lose mobility. Hence, it is expected that different Maillard reaction model systems would possess an optimal a_w for maximum reactivity. What is more, different a_w values lead to the formation of diverse volatiles. Methylpyrazine, for example, exhibits a maximum at a_w 0.65-0.75, while the generation of 2-ethylthiazole formation decreases with increasing a_w.[60]

The pH of the system plays a crucial role in Maillard reactions, since both carbonyl and amine groups have the potential to be charged or uncharged depending on the hydrogen ion concentration of the system. Generally, it has been stated that the rate of browning increases with an increase in pH (up to pH 10).[84] At high pH, a larger percentage of amino groups of the lysine residues of the protein are in the unprotonated form and therefore, more lysine residue can react with reducing sugars and vice versa. The relative protonation of the amino groups is critical, because the initial step of non-enzymatic browning (the condensation of the carbonyl and the amine compounds) is highly pH dependent. Also, the degradation of the Amadori rearrangement products depends on the pH of the system.[2] The pH of the system exerts a crucial effect on the Maillard reaction when the Amadori compound has been formed. At that point, it determines the extent to which reactions proceed by their enolisation, either 1,2- enolisation at low pH or 2,3-enolisation at higher pH. Small changes in the pH of a food product may significantly change the aroma profile after heating.[85] Meynier and Mottram for instance investigated the effect of pH on the formation of volatile compounds in meat-related model system. At low pH, furfural was a major product whereas nitrogen-containing compounds, such as the pyrazines, were detected at higher pH.[86] In addition, the amount of volatile compounds produced during the Maillard reaction depends on the pH of the system. According to Leahy and Reineccius, the rate of pyrazine formation is 500 times higher at pH 9.0 as compared to at pH 5.0 in the same system.[87]

Studies of the Maillard reaction between individual amino acids and sugars have provided a detailed understanding of the pathways for the formation of flavor compounds. However, real foods are much more complex systems and therefore, the provided data of amino acid – sugar models may only be applied to a certain extent. Peptides have been studied less in Maillard model systems. However, it is known already that the reactivity of amino acids does not necessarily remain the same when it is part of a peptide.[8] Ogasawara et al.[88] created Maillard model systems using peptides from 1000 to 5000 Da and xylose, and then evaluated the flavor properties using a sensory panel, yet characteristic volatiles were not identified. Liu et al.[89] studied some sensory characteristics of Maillard reaction products from soy protein hydrolysates of different molecular weights and xylose. The authors reported caramel-like and soy sauce-like characteristic odors in model systems containing peptides of a molecular weight from 1000 to 5000 Da. Further, these Maillard reaction products were analyzed by means of GC-MS and the results reported more than 50 compounds, several of which were nitrogen containing heterocycles.[89] Guo et al.[90] had a similar approach to the work previously mentioned but used *Brassica sp*. as a source of protein to obtain the hydrolysate. The study was focused on the presence of meat-like flavors and the influence of different factors in their generation using model systems that contained *Brassica sp*. proteins at six hydrolysis degrees,

cysteine and xylose. These model systems demonstrated that among hydrolyzed proteins and xylose the overall amount of detected volatile compounds (predominantly cooked-meat-like aromas) was higher at pH 4 than at pH 6 and 8 at temperatures of 160 – 180 ^{o}C. On the other hand, the authors found that the formation of nitrogen-containing heterocycles, such as pyrazines and thiazoles was favored at a higher pH. Afterwards, the reacted mixtures were presented for evaluation by a trained sensory panel to determine their acceptability, and later analyzed by means of GC-MS to identify the volatiles that were produced. The authors detected 132 different compounds in the GC-MS analysis of their different reaction mixtures. However, they reported a small list of the sixteen most abundant ones, nine of which were nitrogen, sulfur and oxygen containing heterocycles.

Concerning these heterocyclic volatile compounds, most investigators agree that alkylpyrazines contribute significantly to the flavor of certain foods, especially due to their low odor threshold (**Table 4**). Moreover, the importance of alkylpyrazines as flavor compounds in roasted food was investigated as early as 1971 by Koehler et al.[91] and further confirmed by several authors.[7,92]

Additionally, some alkylpyrazines are specific Maillard reaction volatiles. Therefore, this literature review will continue focusing on the flavor properties of these pyrazines and the formation mechanisms.

Table 4. Odor threshold of some pyrazines, adapted from Belitz, Grosch and Schieberle[83]

Pyrazine	Aromatic description	Odor threshold value (µg/l; water)
Trimethylpyrazine	Earthy	90
2-Ethyl-3,5-dimethylpyrazine	Earthy, roasted	0.04
2-Ethenyl-3,5-dimethylpyrazine	Earthy, roasted	0.1
2-Ethyl-3,6-dimethylpyrazine	Earthy, roasted	9
2,3-Diethyl-5-methylpyrazine	Earthy, roasted	0.09
2-Ethenyl-3-ethyl-5-methylpyrazine	Earthy, roasted	0.1
Acetylpyrazine	Roasted corn	62
2-Isopropyl-3-methoxypyrazine	Potatoes	0.002
2-sec-Butyl-3-methoxypyrazine	Earthy	0.001
2-Isobutyl-3-methoxypyrazine	Red pepper	0.002

1.6 Pyrazines: occurrence and aroma value in different food products

In 1973 and 1992, Joseph Maga wrote two extensive reviews regarding the occurrence of pyrazines in food products such as coffee, popcorn, roasted pecan nuts, roasted peanuts, fried and boiled beef, potato chips, rice products, frozen cod and krill, tomato, onion, potato and other raw vegetables.[7,92]

It is important to differentiate alkylpyrazines from methoxypyrazines since the second ones can be present naturally in food such as grapes and bell peppers. They are also present in cheese and grape products like wine.[93-96] Meanwhile, alkylpyrazines are mostly reported in food products which were exposed to different heat treatments such as baking, roasting, pressure cooking or boiling. An early conclusion from the authors was that most of the available pyrazines are found in food that is typically toasted or roasted.[7,92] However, it is important to mention that the same reviews also show that several pyrazines occur in food products that were exposed to a hydrolysis treatment such as fermentation or enzymatic hydrolysis. These food products included for example hydrolyzed soy protein, fermented cocoa products, soy sauce and various cheeses, chocolate malt, black malt (used in beer brewing), and bakery products. The variety and abundance of pyrazines are considerably different for various food products. However, the majority can be considered as key odorants.[83] For example, 2-ethyl-3,5-dimethylpyrazine possesses a threshold value of 0.04 µg/kg (**Table 4**), but its concentration in coffee and peanuts is higher than to 1 mg/kg, which makes this pyrazine a key odorant in coffee.[59,97-99]

It is possible to find several other food products which contain pyrazines, nevertheless, a small selection of them will be discussed, emphasizing those in which peptides are abundant, as peptides were suggested to be important flavor precursors.

Chen et al.[100] described the existence of tetramethylpyrazine in Chinese black vinegar, an aged fermented product made from rice, wheat, millet, sorghum, or a combination of these. However, it is not yet known which is the responsible formation mechanism.

Fan et al.[14] identified pyrazines in different Chinese liquors made on the basis of fermentation of sorghum or a mixture of wheat, barley, corn and rice. In this case, the authors stated that the pyrazines could be formed via the Maillard reaction between saccharides and amino groups of protein, peptides or free amino acids. Additionally, it was suggested that these pyrazines could be generated via an unknown microbiological pathway in Chinese liquors during the fermentation step.[14]

Curioni et al.[19] studied several volatiles in different types of cheese and determined their contribution to the overall aroma of these products. Pyrazines have been claimed as important contributors to cheese flavor. For example, 3-isopropyl-2-methoxypyrazine is important in the formation of an earthy soil character in British farmhouse cheddar[93] and 2-ethyl-3,5-dimethylpyrazine and 2,3-diethyl-5-methylpyrazine are considered key odorants of gruyere cheese.[101]

Qian et al.[17] isolated the volatile compounds in parmigiano-reggiano by solvent extraction/high vacuum distillation, followed by separation into acidic, basic, water-soluble and neutral fractions. It was found that the most intense aroma compounds in the basic fraction were 2,6-dimethylpyrazine,

2,3-dimethylpyrazine, trimethylpyrazine, 5-ethyl-2-methylpyridine, 3-ethyl-2,5-dimethylpyrazine, 5-ethyl-2,3-dimethylpyrazine and 2,3,5-trimethyl-6-ethylpyrazine. Moio et al.[102] identified 2,3-dimethylpyrazine, 2,6-dimethylpyrazine, trimethylpyrazine, 3-ethyl-2,5-dimethylpyrazine, and 2-methyl-3,5-diethylpyrazine in grana padano cheese and reported that these compounds have nutty and roasted aromas.

Most authors agree with Morgan, Liardon, Griffith and Hammond[103-105] that the pyrazines may be formed either via the Maillard reaction or through the ambient temperature reaction of microbial metabolites in cheese.

It is important to report the occurrence of pyrazines in cocoa products which typically undergo a fermentation process. According to Maga et al.[7], 10% of about 400 of the compounds identified in the cocoa-aroma fraction are pyrazines. During cocoa fermentation, free amino acids and peptides are formed by proteolytic enzymatic reactions of aspartic peptidases and carboxypeptidases on proteins[106-107], and the reducing sugars, fructose and glucose, are the products of sucrose hydrolysis due to the action of invertase.[108] Hashim and Chaveron[109] detected methylpyrazine, 2,5-dimethylpyrazine, 2,6-dimethylpyrazine, 2,3-dimethylpyrazine, 2,3,5-trimethylpyrazine and 2,3,5,6-tetramethylpyrazine during cocoa fermentation. However, none of them was present in the unfermented beans.[48]

Pyrazines are studied as well in order to evaluate their potential toxicity since they are produced in in several products. Burdock et al.[110] presented a safety assessment of 2-ethyl-3,(5 or 6)-dimethylpyrazine as a food ingredient concluding its food flavoring use is safe at the present user levels as well as for other analog pyrazines. Additionally, a complete safety evaluations of 41 different pyrazines derivatives was done by the European Food Safety Authority (EFSA).[111] The evaluation was mainly focused in genotoxicity (in vitro / in vivo) and intake. According to their safety evaluation summary, pyrazines are safe for all studied cases.[111]

1.7 Formation of Pyrazines

In general terms, pyrazines are formed as the result of a condensation reaction of two α-aminoketones forming a dihydropyrazine that oxidizes to the corresponding pyrazine (**Scheme 5 part II**). Nevertheless, several mechanisms lead to the formation of α-aminoketones (**Figure 3**). Hence, it is probable that in food products, one or more mechanisms act simultaneously to generate pyrazines.

The formation of pyrazines was mainly investigated in model systems containing free amino acids.[4,112-116] However, the formation of pyrazines from peptides and proteins has been reported as well.[11,27-30,117-121] The different pathways leading to the formation of α-aminoketones and their further condensation into pyrazines are described in detail in the following subsections. Additionally, it has been reported that free ammonia can react with glucose forming aldosylamines, which can

undergo enolization to form α-aminoketones. However, glucose fragmentation products such as glycoaldehyde or pyruvaldehyde can condense with ammonia to form α-aminoketones.[75] Therefore, different mechanisms involving the release of ammonia from peptides and proteins are also included.

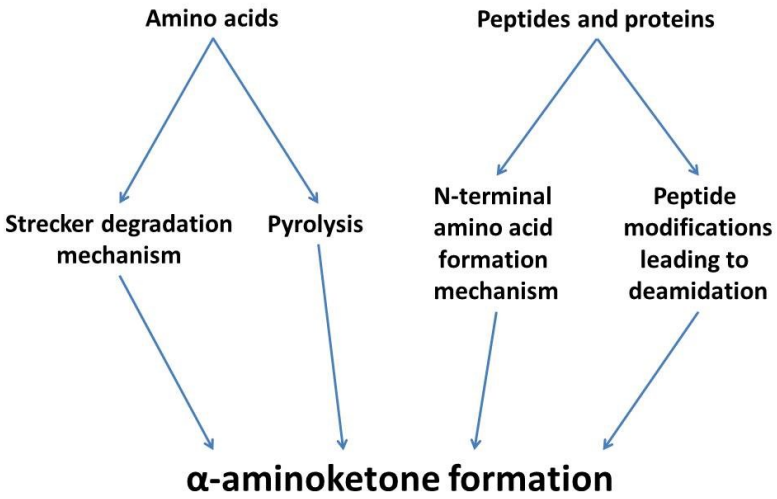


Figure 3. Overview of different degradation mechanisms leading to the formation of α-aminoketones.

1.7.1 Formation of alkylpyrazines in reactions with free amino acids via the Strecker degradation pathway

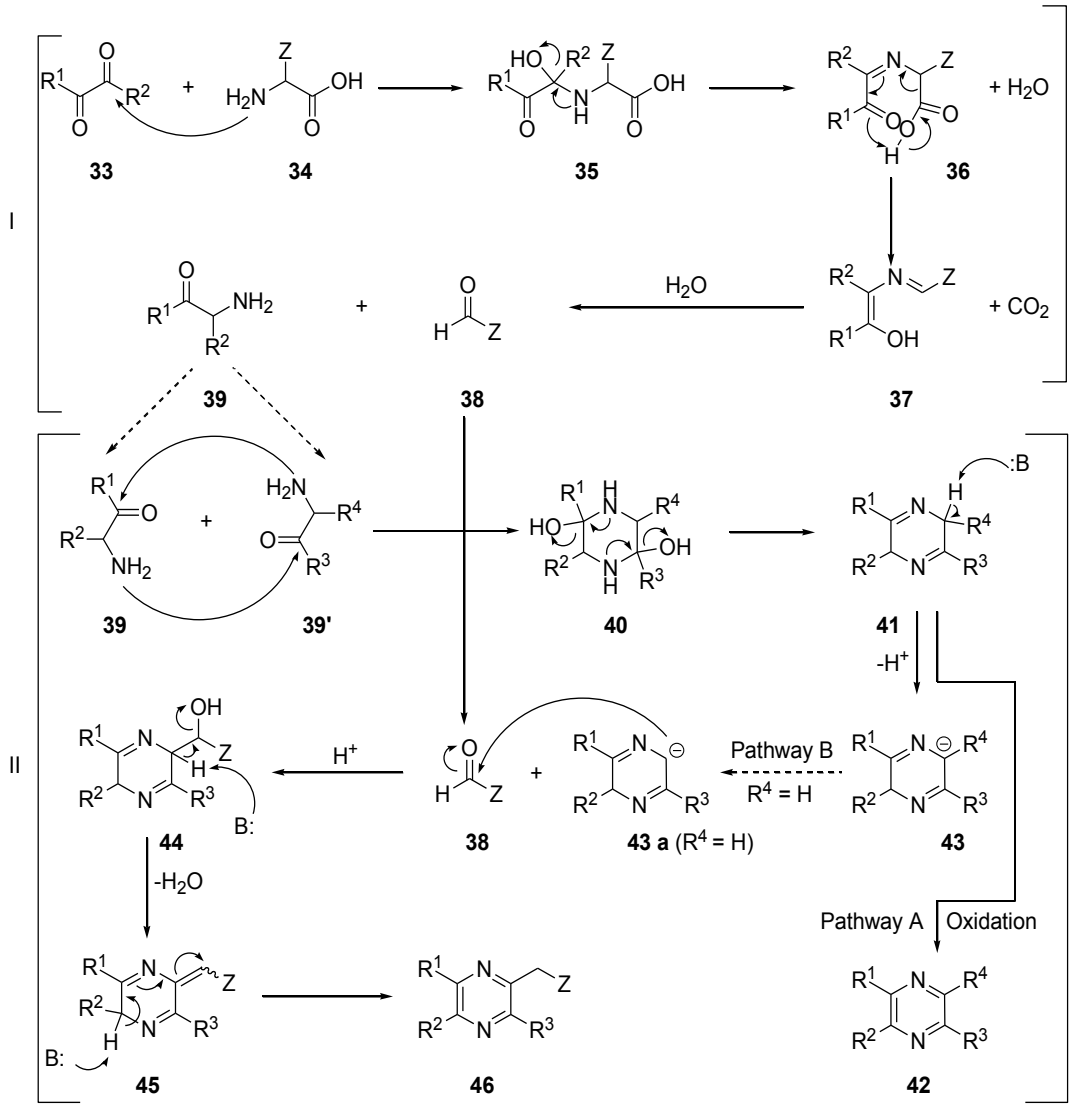
The first step of pyrazine formation via the Maillard reaction implies the reaction of a dicarbonyl compound **33** (**Scheme 5 part I**) produced by sugar degradation and an amino acid **34**, which leads to the formation of a Strecker aldehyde **38** and an α-aminoketone **39**. A crucial step in this degradation reaction is the decarboxylation of the intermediate **36** via a cyclic transition state. The second step in the pyrazine formation involves two possible pathways which start with the condensation reaction of two α-aminocarbonyl compounds **39** and **39'** with the formation of a dihydropyrazine **41**. This dihydropyrazine can react in two distinct ways. The dihydropyrazine can oxidize spontaneously with air to the corresponding pyrazine **42** (pathway A). Alternatively, after deprotonation, the dihydropyrazine anion **43a** can react with a carbonyl compound in an aldol-type reaction (pathway B). Whenever this reacting carbonyl compound is a Strecker aldehyde **38**, amino acid specific pyrazines **46** are formed (**Scheme 5 part II**). Some examples of amino acid specific pyrazines are depicted in **Scheme 6**.

Several authors studied the formation of pyrazines in model systems containing free amino acids and glucose in a wide variety of conditions. The results of such investigations allowed evaluating the reactivity of different amino acids, the factors that affect the formation of pyrazines, and the formation mechanisms of numerous pyrazine precursors.

Initially, the formation of pyrazines was studied in simple aqueous model systems, containing only one amino acid and glucose, or glucose reacting with ammonia. These experiments were conducted under various conditions, with temperatures ranging between -5 ^{o}C and 140 ^{o}C and reaction times from 15 minutes to 30 days. Shibamoto and Bernhard[75,116,122] found a large variety of pyrazines in their model systems which revealed the importance of the glucose degradation products in the formation of different alkylpyrazines.

Koehler and Odell[114] studied a number of factors affecting the formation of pyrazines. They compared a system of glucose and asparagine or other amino acids. In this experimental setup, the reagents were dissolved in diethylene glycol and water and the reacting conditions were a temperature of 120 ^{o}C for 24h. It was found that the pyrazine yields with asparagine were five times higher than in models containing glycine, alanine, lysine or aspartic acid. At the same time, the authors replaced glucose by potential fragmentation products such as glyoxal, butane-2,3-dione and hydroxyacetone. The experiments containing glyoxal yielded mainly the unsubstituted pyrazine, but some methylpyrazine was also found. Butane-2,3-dione gave relative high amounts of tetramethylpyrazine, and hydroxyacetone was the main precursor of all dimethylpyrazine isomers.

Novotny et al.[123] studied the formation of α-hydroxycarbonyl and α-dicarbonyl compounds during degradation of monosaccharides and observed formation of glycoaldehyde, acetol, 1,3-dihydroxyacetone, glyceraldehyde, lactaldehyde, acetoin, 1-hydroxybutan-2-one, glyoxal, methylglyoxal, butane-2,3-dione, 1-hydroxybutane-2,3-dione, ethylglyoxal and pentane-2,3-dione. Sugar degradation products and fission products of the rearranged sugars which are obtained during the intermediate stage of the Maillard reaction, are important precursors for the formation of pyrazines. Additionally, following the mechanism pathway A of **Scheme 5**, it is possible to rationalize the formation of pyrazines according to the reacting α-diones (**Scheme 7**).

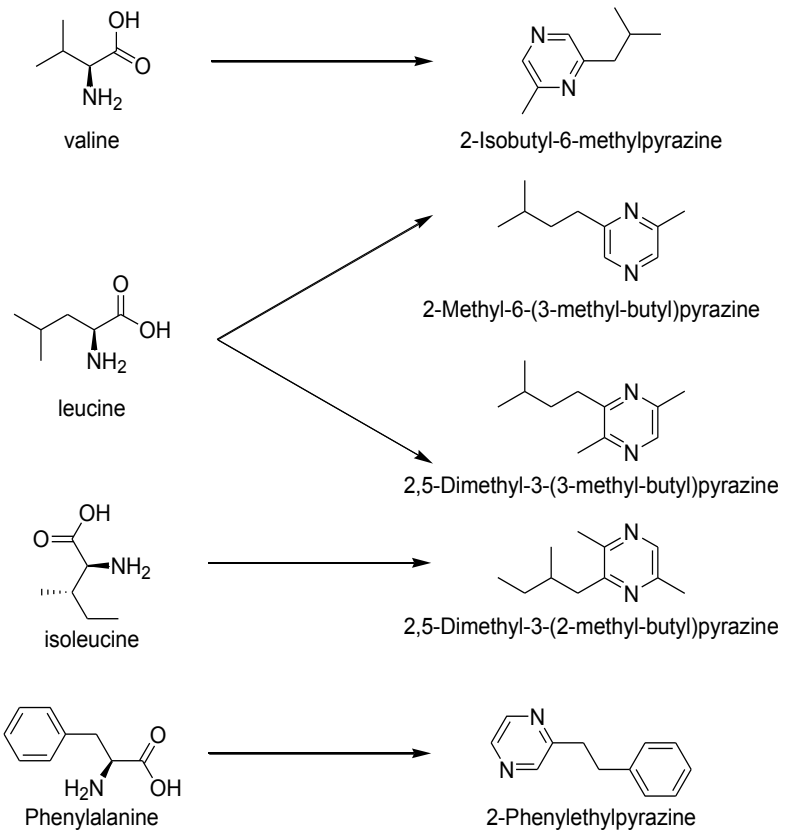


Scheme 5. Strecker degradation (part I) and pyrazine formation (part II), adapted from Van Lancker et al.[120]

Rizzi et al[124] used a model system containing methylglyoxal and glycine under continuous reflux and expected to find 2,5-dimethylpyrazine and 2,6-dimethylpyrazine, but trimethylpyrazine was found as well. The authors attributed the extra methyl group of trimethylpyrazine to aldol condensations of appropriate intermediates with formaldehyde or acetaldehyde.

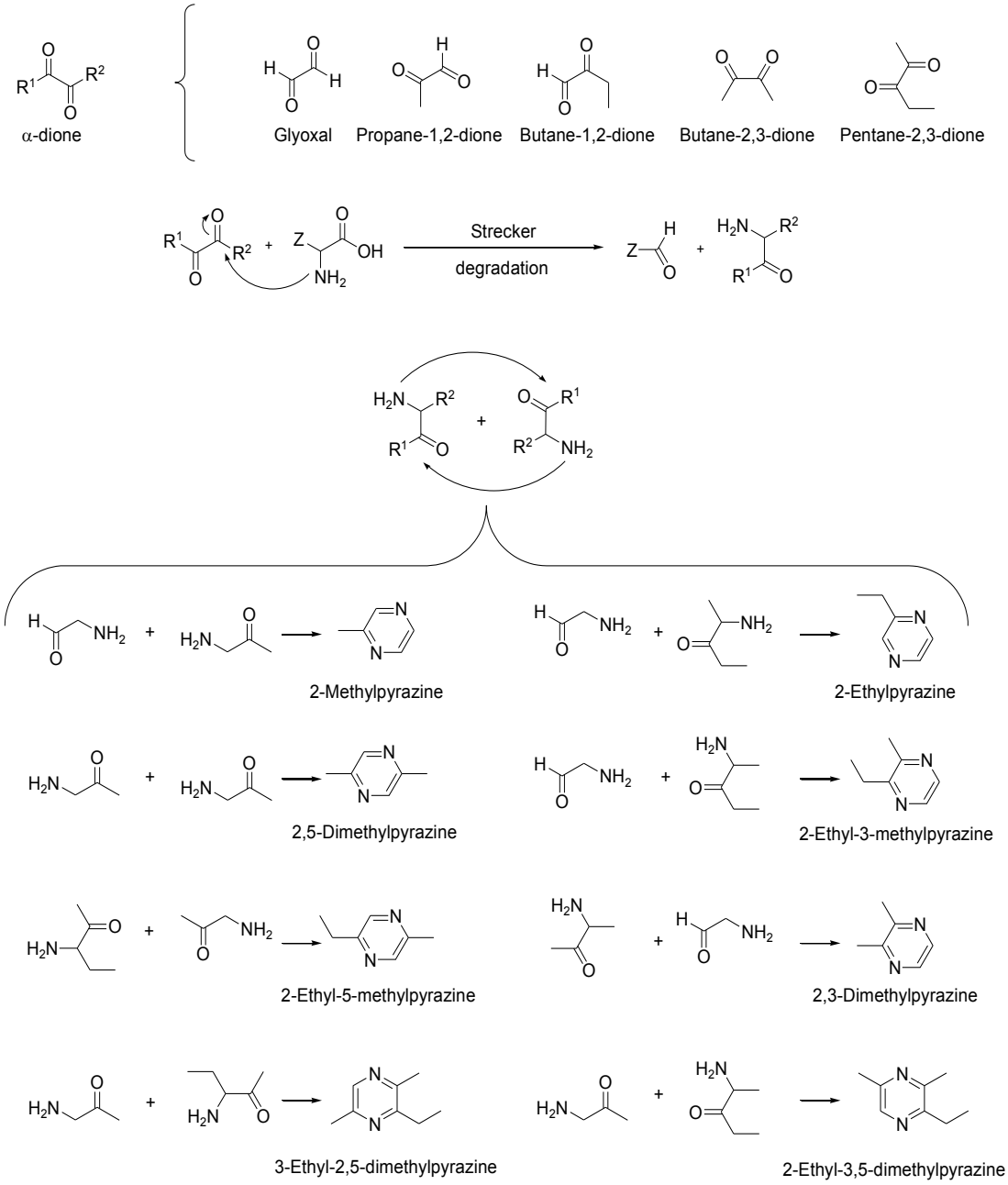
Other amino acids can lead to an unexpected formation of pyrazines. Hwang et al.[113] studied the reaction of α-^{15}N-labelled free lysine with glucose, more specifically the relative contribution of the α- and ε-amino groups of lysine in pyrazine production. The reactions were carried out under dry heating conditions at different temperatures ranging from 100 – 180 ^{o}C for 1 h. The authors found that about 40% of the nitrogen of the unsubstituted pyrazine was incorporated from the ε-amino group. Additionally, 2,5-dimethylpyrazine and trimethylpyrazine incorporated nitrogen exclusively

from the α-amino group. Hence, it was suggested that the α-amino group generated the α-aminoketones through Strecker degradation while the ε-amino group forms α-aminoketones by an intramolecular rearrangement followed by hydrolysis of the imine.



Scheme 6. Amino acid specific pyrazines.

Chen and Ho et al.[125] compared the formation of pyrazines in aqueous model systems of serine, threonine and glutamine with ribose, glucose and fructose, each one of the mixtures had a pH of 8.00. The samples were further heated at 160 ^{o}C for 2 h. The model systems containing glutamine yielded the highest amounts of total pyrazines. The authors attributed this enhanced alkylpyrazine formation to a release of ammonia due to deamidation of glutamine.[125]



Scheme 7. Formation of α-aminoketones from α-diones and their predictive condensation to form different pyrazines.

Hwang et al.[4] described the relative reactivity of glutamine, glutamic acid, asparagine, aspartic acid, lysine, arginine, phenylalanine and isoleucine in pyrazine formation. An experiment was done to monitor how the amino acids competed in systems containing ^{15}N labeled glycine as reference. The amino acid – glucose mixtures were freeze-dried and then incubated to a final moisture content of 12 – 14%. The samples were then transferred to a reaction vessel and heated at 180 ^{o}C for 1 h. The

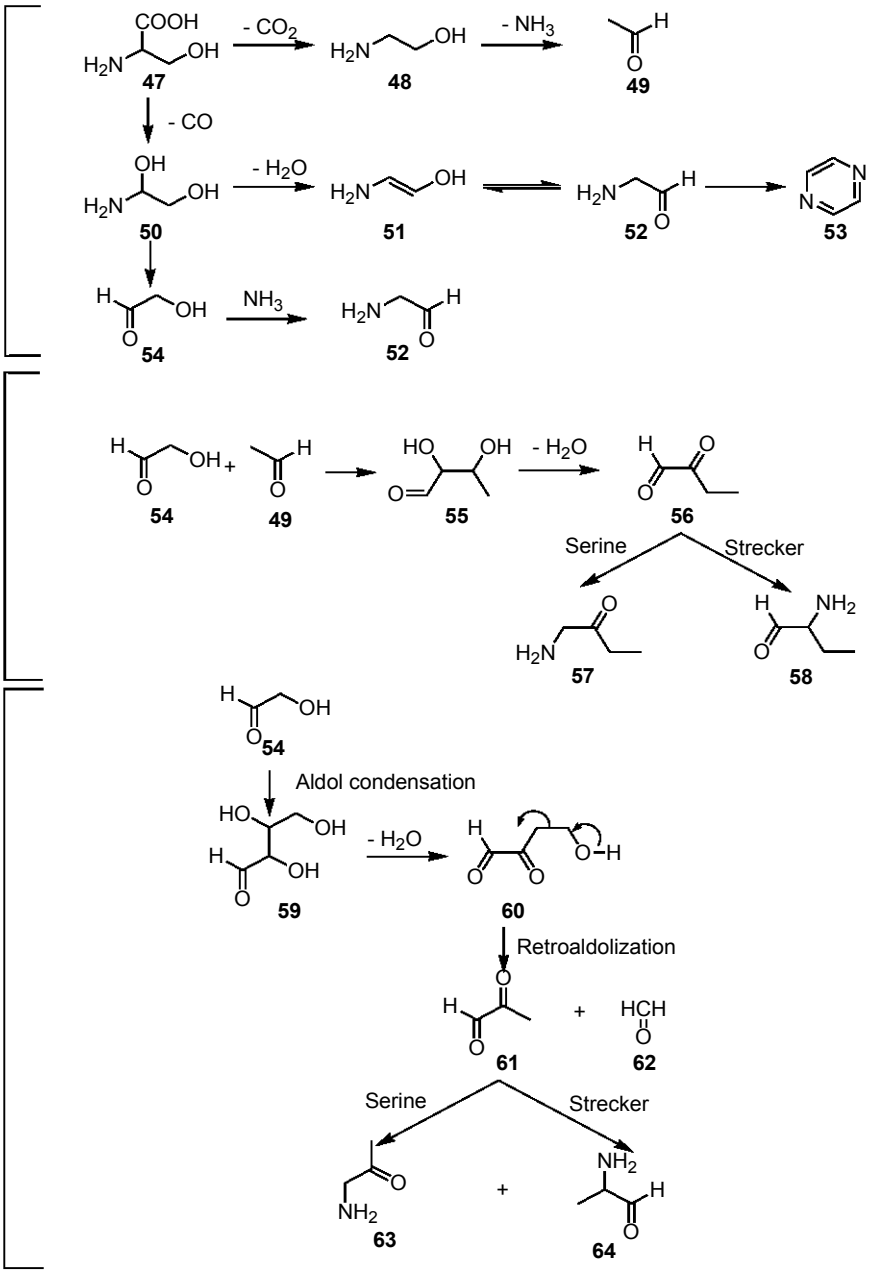
authors found that most of the pyrazines were present regardless of amino acid type. In the competition experiment, it was found that in the presence of glycine, glutamine and glutamic acid exhibited a low contribution, while asparagine was the highest contributor to pyrazine formation. By comparing the total yields of pyrazines generated in the different reaction mixtures, it was found that the reaction mixture containing lysine had the highest yield, while the reaction mixture containing arginine had the lowest. The results imply that lysine has a catalytic effect on glycine while arginine has an inhibitory effect.

1.7.2 Alkylpyrazines formation from pyrolysis of serine and threonine

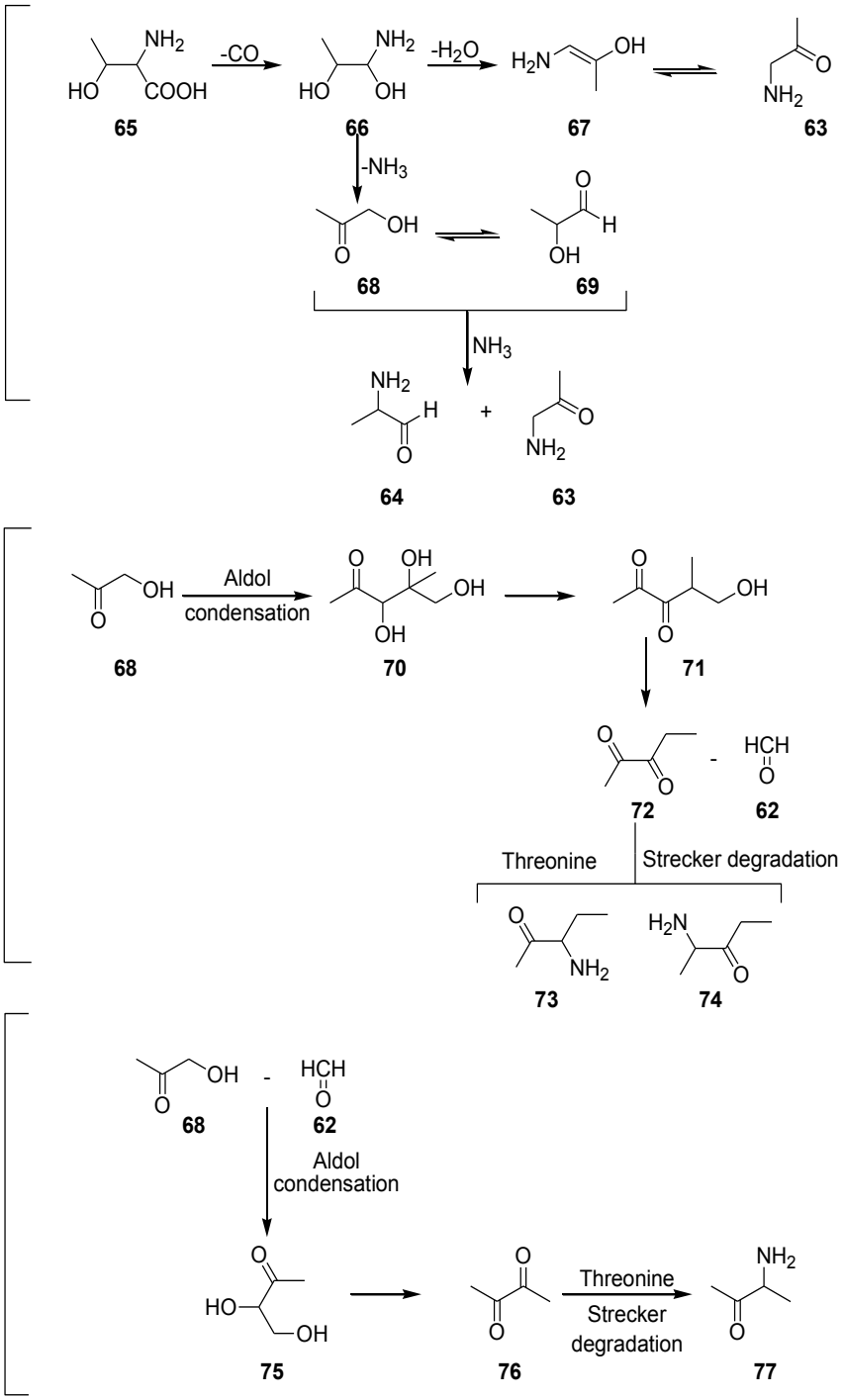
Alkylpyrazines are normally generated through Strecker degradation involving α-amino acids and α-dicarbonyl compounds **30**, generated as cleavage products of rearranged sugars from the initial stage of the Maillard reaction, or due to caramelization and degradation of sugars. Shibamoto et al.[75] described the ammonia/acyloin pathway, a reaction in which acyloins are converted to α-aminocarbonyls and their further condensation towards alkylpyrazines. Both pathways require a carbohydrate source to provide the degradation products, either α-dicarbonyl compounds or acyloin compounds.[126] However, the formation of alkylpyrazines from serine **47** and threonine **65**, without a carbohydrate source, has been reported as well.[126-128] Yaylayan et al.[127] detected different alkylpyrazines in model systems containing ^{13}C-labeled serine. Further, it was found in this study that pyrolysis of serine **47** gives rise to the formation of α-hydroxyacetaldehyde **54**, methylglyoxal, butan-2,3-dione and other carbonyl compounds which are able to react with other molecules of serine **47** to produce different alkylpyrazines.

Shu et al.[126] suggested that the formation of alkylpyrazines from serine **47** could be due to the mechanism depicted in **Scheme 8**. The first step involves decarbonylation followed by dehydration to form the precursor α-aminoacetaldehyde **52**, which is dimerized to form pyrazine **53**. Meanwhile, decarbonylation followed by deamination forms α-hydroxyacetaldehyde **54** which can react with ammonia and form α-aminoacetaldehyde **52**. Decarboxylation of serine **47** followed by deamination leads to the formation of acetaldehyde **49**. These reactive intermediates participate further in the reaction. For example, the aldol condensation of α-hydroxyacetaldehyde **54** and acetaldehyde **49**, followed by dehydration generates butan-1,2-dione **56**, which can react with another molecule of serine **47** to form 1-amino-butan-2-one **57** and 2-aminobutanal **58**. α-Hydroxyacetaldehyde **54** can generate 1-amino-propan-2-one **63** and 2-aminopropanal **64** via aldol condensation and retroaldol reaction. Ethylpyrazine can be obtained then by the combination of α-aminoacetaldehyde **52** and 1-amino-butan-2-one **57**. Further, 2,6-dimethylpyrazine can be formed by combination of 1-amino-

propan-2-one **63** and 2-aminopropanal **64**. The combination of 1-amino-butan-2-one **57** and 2-aminopropanal **64** or 2-aminobutanal **58** and 1-amino-propan-2-one **63** can generate 2-ethyl-6-methylpyrazine. Additionally, the combination of α-aminoacetaldehyde **52** and 1-amino-propan-2-one **63** or α-aminoacetaldehyde **52** and 2-aminopropanal **64** can generate methylpyrazine.



Scheme 8. Formation of pyrazine precursors from the pyrolysis of serine, adapted from Shu et al.[126]



Scheme 9. Formation of pyrazine precursors from pyrolysis of threonine, adapted from Shu et al.[126]

Scheme 9 depicts the pathways involving threonine **65** that lead to the generation of alkylpyrazine precursors. These mechanisms are similar to those of serine **47**, as for example decarbonylation and dehydration of threonine **65** form precursor 1-amino-propan-2-one **63** which is dimerized to form

2,5-dimethylpyrazine. Aldol condensation of 1-hydroxy-propan-2-one **68** followed by its retroaldol reaction and Strecker degradation generates the precursors 3-amino-pentan-2-one **73** and 2-amino-pentan-3-one **74**. 2-ethyl-3,6-dimethylpyrazine can be formed by the combination of 1-amino-propan-2-one **63** and 3-amino-pentan-2-one **73**, and 2-ethyl-3,5-dimethylpyrazine by the combination of 1-amino-propan-2-one **63** and and 2-amino-pentan-3-one **74**. Finally, 1-hydroxy-propan-2-one **68** and formaldehyde **62** can react to form the precursor 3-amino-butan-2-one **77**; the combination of 1-amino-propan-2-one **63** and 3-amino-butan-2-one **77** can form trimethylpyrazine.

1.7.3 Formation of alkylpyrazines in reactions between peptides and carbohydrates

Several studies have focused on the differences of flavor and aroma formation during Maillard reactions with peptides as compared to free amino acids. From these studies it can be concluded that, in general, both systems generate similar aroma compounds but in different ratios and quantities. Of the reactions with peptides, the formation of pyrazines, furans, pyrrolizines, thiazoles and thiophenes has been described. However, the following section will focus mainly on the formation of pyrazines since their importance was discussed already.

Oh et al.[11] studied the generation of volatile compounds in aqueous model systems containing glucose, glycine, diglycine, triglycine and tetraglycine at a temperature of 180 ^{o}C for 2 h. The authors found that glycine and triglycine generated more pyrazines than diglycine and tetraglycine. Further, it was suggested that glycine reacts directly with glucose to form pyrazines, while triglycine is degraded into glycine and diglycine through the formation of diketopiperazine. Tetraglycine is probably degraded into diglycine and further degraded to glycine to generate pyrazines. Nevertheless, this process would have a high activation energy. Additionally, the direct degradation of the peptides without the intermediate formation of a diketopiperazine cannot be disproved. In a subsequent work, Oh et al.[117] investigated the volatile compounds generated in the aqueous Maillard model systems containing Pro-Gly, Gly-Pro, and a mixture of glycine and proline with glucose. All model systems were heated in an oil bath at different temperatures between 130 ^{o}C and 180 ^{o}C. The authors reported that mainly pyrrolizidines and pyridines were generated in model systems at 130 ^{o}C. Pyrrolizines have been reported as proline-specific Maillard products. However, when the temperature was increased to 180 ^{o}C, pyrazines and pyrrolizines were generated in similar amounts.

Lu et al.[118] found similar results in various aqueous model systems containing glycine, diglycine, triglycine and glucose, which were heated at 160 ^{o}C for 1h in an oil bath. In this case, the major volatiles produced were trimethylpyrazine and 2,5-dimethylpyrazine. The overall reactivity decreased

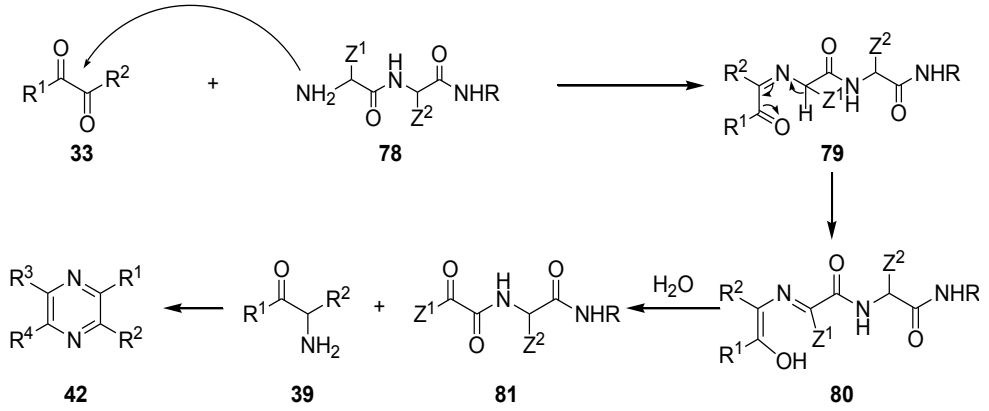
in the order glycine > triglycine > diglycine, and the higher reactivity of triglycine than of diglycine in alkylpyrazine formation may be related to the availability of free glycine during the reaction due to a high hydrolysis rate.

Zhang et al.[119] presented results suggesting that peptides directly contribute to the formation of volatiles in Maillard reactions. The authors isolated peptides from a casein hydrolysate, characterized them by gel permeation chromatography and then made them react with glucose to identify the generated volatiles. The obtained compounds in the reaction of the hydrolysate and glucose were carbohydrate-derived carbonyls, furans, large quantities of 2-methylpropanal, 3-methylbutanal and phenylacetaldehyde (Strecker aldehydes of valine, leucine and phenylalanine) and pyrazines. It was noted by the authors that the detected amounts of Strecker aldehydes **36** could be due to peptide hydrolysis during the selected conditions, as the hydrolysate contained very small amounts of free amino acids. Yang et al.[129] investigated the mechanism of peptide bond cleavage and the generation of volatiles for the Maillard reaction using ten different oligopeptides and xylose in model systems that were heated at 132 ^{o}C during 90 min. Valuable conclusions regarding how peptides are hydrolyzed depending on their sequence were obtained. Some peptide bonds were considered to be more stable than others. For example, it was suggested that the bonds between glutamic acid and lysine are resistant to cleavage due the formation of hydrogen bonds. Regarding the formation of volatiles, pyrazines were not found in this experiment, which was contrary to expectations. However, the authors attributed these phenomena to the low pH and temperature conditions.

As observed, the initial investigations regarding formation of pyrazines in peptide containing model systems focused on the hydrolysis of the peptides and the subsequent reaction of the free amino acids. However, peptides were not considered as the initial reagents leading to the formation of pyrazines. Van Lancker et al.[120] studied the formation of pyrazines in lysine-containing dipeptides during the Maillard reaction. Several model systems, containing eight different dipeptides, were reacted with glucose, glyoxal and methylglyoxal with the purpose not only of evaluating the pyrazine generation, but also to elucidate their mechanism of formation. In all cases, the different aqueous mixtures were heated at 130 ^{o}C for 2h in an oil bath. The selected dipeptides were compared to the free amino acids that make up the sequence of the aforementioned peptides in terms of their capacity to generate pyrazines. It was found that more pyrazines were produced in reactions with dipeptides than in reactions with free amino acids, with the exception of the dipeptide Lys-Leu. It could also be observed that pyrazines comprised a much bigger portion of the total volatiles produced in the case of the dipeptides as compared to their corresponding free amino acids, except for the case of amino acid specific pyrazines such as 2,5-dimethyl-3-(3-methylbutyl)pyrazine from leucine. Besides glucose, similar model systems were made with methylglyoxal and glyoxal with the

objective to limit the pyrazine spectrum and thus, to have a clearer view in the mechanism involved in pyrazine formation mechanisms. Van Lancker et al.[120] hypothesized a reaction mechanism for the formation of α-aminoketones **39** from a dipeptide **78** and an α-dicarbonyl compound **33** which is depicted in **Scheme 10**.

Peptides cannot follow the typical Strecker degradation due to the absence of a free carboxyl group at the α-carbon with respect to the free amino group, making the decarboxylation impossible. In accordance to the reaction with free amino acids, the reaction of the α-dicarbonyl compound **33** with the dipeptide starts with the formation of an α-ketoimine **79**. Afterwards, deprotonation occurs at the α-position of the amide moiety resulting in a 1,5-*H*-shift leading to enolization of the carbonyl group of intermediate **80**. Hydrolysis of the imino moiety of 4-hydroxy-2-azadiene **80** produces the α-aminoketone **39** and instead of forming the Strecker aldehyde, a complex α-ketoamide **81** is formed.



Scheme 10. Hypothetical formation mechanism of α-aminoketones in the reaction of peptides with α-dicarbonyl compounds. Adapted from Van Lancker et al.[120]

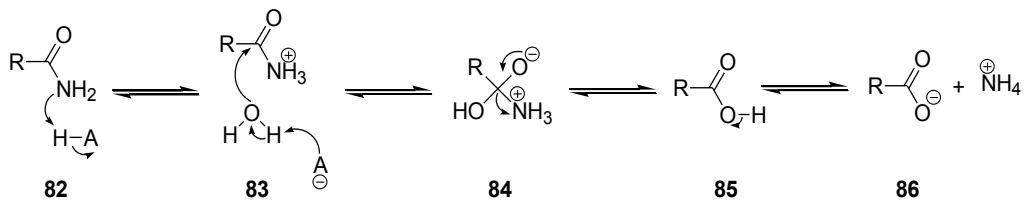
Van Lancker et al.[121] not only showed that peptides are capable of producing pyrazines and other flavor compounds, but also that the *N*-terminal amino acid of the peptide has a strong influence on the generation of pyrazines. For example, the formation of 2,5-dimethylpyrazine was low for proline, leucine and valine at the *N*-terminus of the peptide, while the production of this pyrazine was enhanced for glycine, alanine or serine. The control experiment showed that unsubstituted pyrazine was produced in higher levels in the case of free amino acids, which was rare with peptides and therefore, the mechanisms responsible for pyrazine generation might be different for peptides, than for amino acids.

1.7.4 Formation of alkylpyrazines as a consequence of peptide modifications

There are several mechanisms in which ammonia is released from the side chains of peptides, particularly when they contain asparagine or glutamine, leading to the modification of peptides. This group of reactions has different mechanism pathways that release ammonia but their name is commonly referred to as nonenzymatic deamidation. In all deamidation reactions ammonia is released. Therefore, one can speculate that the generated ammonia would react with reducing sugars in Maillard reactions almost in the same way as free amino acids.[75,122] At the same time, in later stages of the Maillard reaction, ammonia can also react with dicarbonyl compounds produced by degradation of sugars to form aminoketones, which are precursors to generate pyrazines.[130]
The most common mechanisms of deamidation on peptides are described below. Further, other factors that affect deamidation reactions have been extensively reviewed and therefore will not be included in the present text.[130-131]

1.7.4.1 Mechanism of acid- and base-catalyzed deamidation

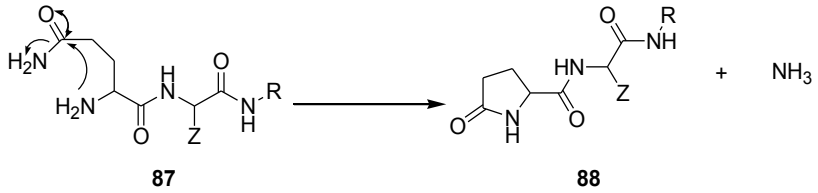
The deamidation of asparagine and glutamine to their corresponding carboxylic acid, occurs in the presence of an acid and a base, depicted as HA and A^- in **Scheme 11**. The first step of the process concerns the protonation of the amide **82** to its conjugated acid **83**. The newly formed conjugated acid **83** can then undergo a nucleophilic attack at the electrophilic carbon of the carbonyl group by the base (A^-) or by a hydroxyl anion generated by the base if water is present. In both cases, an oxyanion intermediate is created which is stabilized when proton donors are present. Next, the tetrahedral intermediate **84** transforms to the respective carboxylic acid **85** by eliminating ammonia and once equilibrium is reached the carboxylate **86** is formed. It is clear that the pH in the medium has an important impact on the reaction rates of the deamidation of proteins in solution as it controls the activation of the amide **82** to its conjugated acid **83** and stabilizes the oxyanion intermediate **84**.



Scheme 11. Mechanism of acid and base catalyzed deamidation

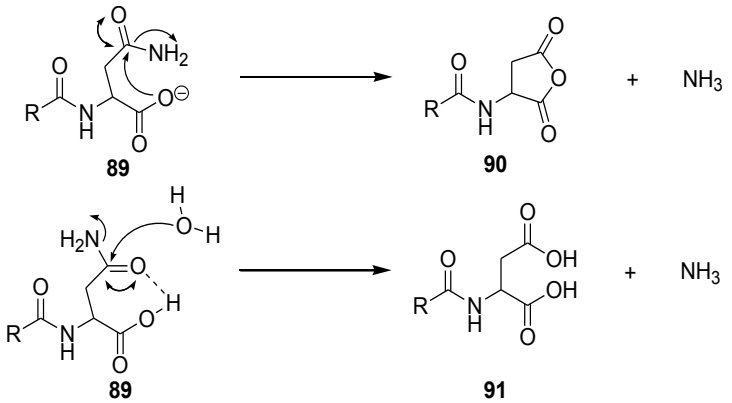
1.7.4.2 Cyclization of glutamine at the *N*-terminal and asparagine at the *C*-terminal

Another pathway of deamidation is cyclization of glutamine, which happens at the *N*-terminus of the peptide **87 (Scheme 12)** by cyclization of the side chain with its own terminal amino group to form an amide bond.



Scheme 12. Cyclization of glutamine at the *N*-terminus and asparagine at the *C*-terminus

Asparagine (**89**) (**Scheme 13**) does not cyclize at the *N*-terminus because the size of the molecule would result in the formation of a four-membered ring. However, it may undergo deamidation at the *C*-terminus. Two possible mechanisms have been reported for this reaction. First, a nucleophilic attack of the carboxylate group at the amide side chain may result in the deamidation of a carboxyl terminal asparagine (**89**) to form anhydride (**90**).[131] Alternatively, it has been suggested that under acidic conditions, the protonated terminal carboxylate group stabilizes the side chain of asparagine (**89**) by hydrogen bonding in a conformation that is susceptible to attack by a solvent nucleophile[131].



Scheme 13. Mechanism for the deamidation of carboxy-terminal asparagine, adapted from Wright[131]

1.7.5 Formation of alkylpyrazines from carbohydrates and proteins

As outlined above, Maga et al.[7,92] wrote two reviews which describe the presence of pyrazines in food, (briefly introduced in **Section 1.7**). These review articles highlight the presence of alkylpyrazines in food products that are known to have high levels of proteins such as beef, casein, milk powder, turkey, eggs, krill, almonds, hazelnuts, rice, coffee beans, barley, walnuts and peanuts. However, several other investigations were published after the last update presented by Maga. Sekiwa et al.[132] reported that various pyrazines compose the characteristic flavor of cooked clam. Buttery et al.[133] reported that pyrazines were generated in high quantities in rice cakes, significantly contributing to their characteristic flavor. Madruga et al. [134-135] studied the volatile flavor profile of grilled goat meat and reported thirteen pyrazines among the volatiles generated. Kwon et al.[136] developed a method for the determination of pyrazines in perilla seed oils, and at the same time, studied the impact of roasting conditions in the generation of these pyrazines. Nicolotti et al.[137] identified several alkylpyrazines in roasted hazelnuts and further studied their formation as a consequence of processing and the origin of the different hazelnuts. Xiao et al.[138] also reported pyrazines as being part of the volatiles generated in raw and dry-roasted almonds.

Natural food products possess a more complex matrix than most of the studied model systems, and for this reason, it is expected that the generation of pyrazines might be the result of several mechanism acting simultaneously. Surprisingly, the formation of pyrazines in model systems containing pure proteins has been investigated much less. Lee et al.[139] has described the formation of pyrazines in model systems containing deamidated but not regular wheat gluten protein. The authors concluded that ammonia released from wheat protein via deamidation participates in the generation of pyrazines.[139] Other investigations suggest proteins as sources of pyrazines such as in the model systems presented by Ferretti, Jing and Jiang. Nevertheless, the experimental conditions these authors used were quite intense, and for such reason it is not possible to neglect thermal hydrolysis of the proteins leading to the formation of pyrazines.[24-26]

There are several other studies regarding Maillard reactions between proteins and sugars at lower temperatures, but the formation of pyrazines was not reported. Moreover, such studies describe the effects of glycation on functional properties of proteins via the Maillard reaction.[23,140-143]

1.8 CONCLUSIONS

The presence of several pyrazines in a wide variety of food has been reported extensively. However, most of the authors reported mainly the presence of pyrazines among other compounds as a result of the Maillard reaction without investigating in depth the mechanisms that may lead to their

formation. Pyrazines are present in fermented and non-fermented food products, indicating that the pyrazine generation can have different precursors.

The formation of pyrazines was investigated in various model systems. As a result, it is known that several pathways can be responsible for the generation of these volatiles. Nevertheless, until now, free amino acids have been considered the main precursors for the formation of pyrazines. However, generally spoken, the content of free amino acids in food is low. Therefore, it is not clear whether free amino acids act as the main as contributors for the generation of pyrazines if their concentration is low. Alternatively, peptides and proteins could be relevant precursors. Peptides, as free amino acids, in general are present in low concentrations as well in food products, except in fermented foods. Hence, since the abundance of free amino acids and peptides is similar, a question arises: Can peptides be effective precursors for the generation of pyrazines? Moreover, another question is if proteins as such can generate pyrazines in realistic heating conditions.

It is clear that for different food products, the precursors for the generation of pyrazines will be different as well. As for example, a fermented soy sauce contains a high amount of free amino acids, it is likely that they contribute significantly. Additionally, for bakery products in general, it is possible that the glutamine present in the wheat proteins can be a source for the pyrazine generation as a result of deamidation. Finally, in meat products, it is clear that protein and peptides are most likely precursors. The formation of pyrazines from free amino acids, and due to deamidation, has been reviewed in detail. Nevertheless, the role of peptides needs to be evaluated as well.

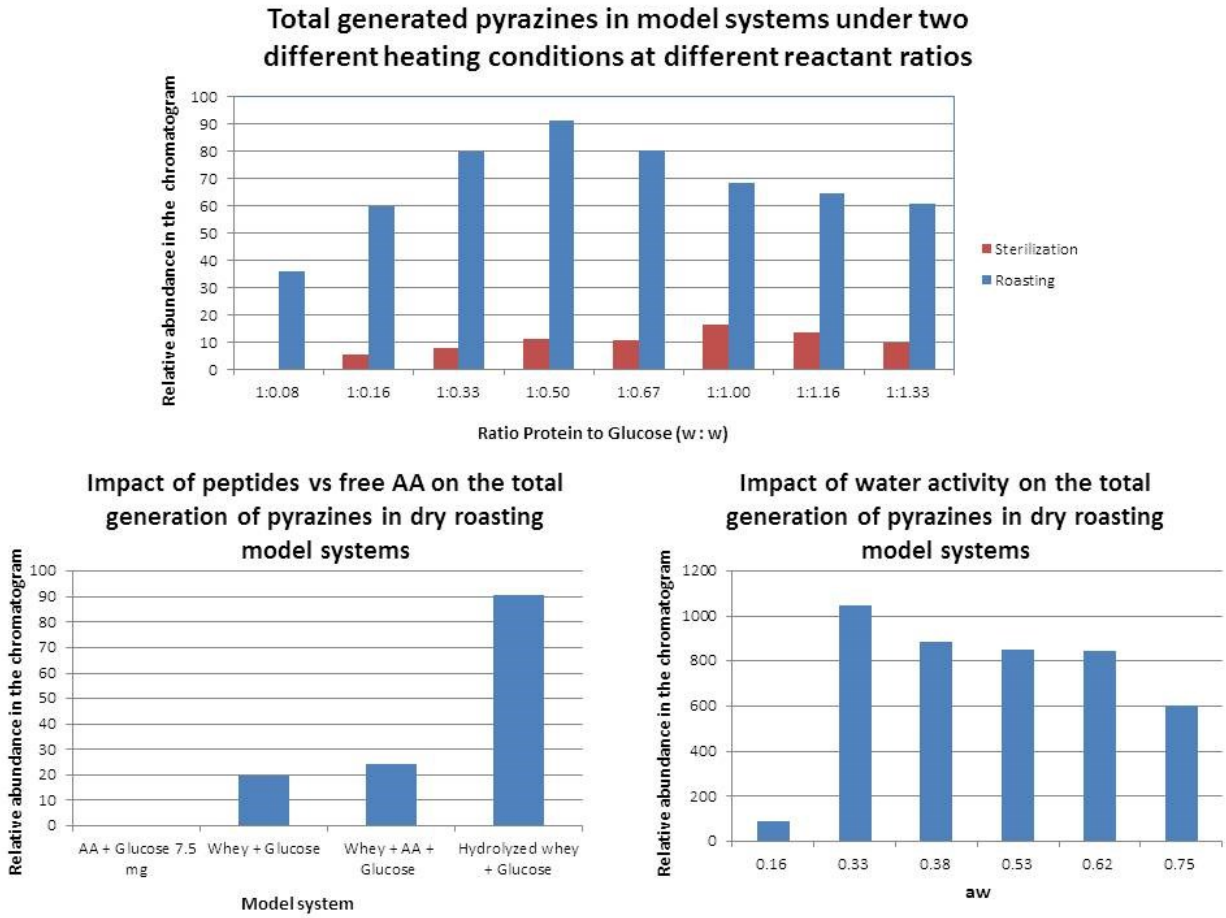
CHAPTER 2

Influence of free amino acids, oligopeptides and polypeptides on the formation of alkylpyrazines in Maillard model systems containing tryptic hydrolyzed whey protein

ABSTRACT

Pyrazines are specific Maillard reaction compounds known to contribute to the unique aroma of many products. Most studies concerning the generation of pyrazines in the Maillard reaction have focused on amino acids, while little information is available on the impact of peptides and proteins. The present study investigated the generation of pyrazines in model systems containing whey protein, hydrolyzed whey protein, amino acids and glucose. The impact of thermal conditions, the ratio of the reagents and the a_w on the pyrazine formation was measured by HS-SPME-GC-MS. The presence of oligopeptides from hydrolyzed whey protein contributes significantly to an increased amount of pyrazines, while, in contrast, free amino acids generated during protein hydrolysis contributed to a lesser extent. The generation of pyrazines was enhanced at low a_w (0.33) and high temperatures (> 120 ^{o}C). This study showed that the role of peptides in the generation of pyrazines in Maillard reaction systems has been dramatically underestimated.

KEYWORDS: peptides; Maillard reaction; fractions; pyrazines; flavor; model reactions; HS-SPME-GC-MS.



Graphic Abstract 1. Summarized information of **Chapter 2**

2.1 INTRODUCTION

The reaction between free amino acids and carbonyl compounds has been extensively studied[8], whereas the Maillard reaction between peptides and proteins with carbonyl compounds has been less investigated.[9-11] This in fact is quite surprising as the level of free amino acids in food is generally very low as compared to the level of peptides and especially proteins.[144]

Oligopeptides have been recognized as important flavor enhancers and precursors of the Maillard reaction.[9,21-23] Moreover, peptides can be flavor enhancers as such (umami taste). In addition, the formation of flavor compounds due to the reaction between peptides and carbohydrates has mainly been studied on model systems containing glutathione[27-30] and glycine-derived peptides such as diglycine, triglycine, and tetraglycine.[11] In these studies, pyrazines were indeed the most abundant volatiles.

Although it has been shown before that peptides may generate pyrazines in the Maillard reaction, their potential contribution to the overall pyrazine formation in food has not been compared to the contribution of amino acids. Therefore, this study aimed to evaluate the impact of trypsinogenic whey hydrolysis products on the formation of pyrazines in a Maillard reaction model, thereby trying to discriminate the roles of free amino acids and peptides generated, respectively. In addition, the impact of other factors such as heating conditions, the ratio of reagents and the a_w on the formation of pyrazines were investigated.

2.2 MATERIALS AND METHODS

2.2.1 Samples and Chemicals

DL–norvaline (99%), L–cysteine (99.5%), L–4-hydroxyproline (99%), DL–valine (99%), DL–alanine (99%), L–tryptophan (99.5%), L–citruline (99%), sarcosine (99%), DL–histidine (99%), L–isoleucine (99.5%), DL–leucine (99%) and glutamine (99,5%) were purchased from Fluka (Sigma–Aldrich, Bornem, Belgium). DL–lysine monohydrochloride (98%), DL–methionine (99%), glycine (99.5%), L–arginine hydrochloride (99%), L–phenylalanine (99%), L–glutamic acid (99%), D-(+)-glucose (99.5%), trypsin from porcine pancreas, trypsin-chymotrypsin inhibitor from glycine max (soybean)*, insulin from bovine pancreas*, insulin chain B oxidized from bovine pancreas*, cytochrome C from equine heart*, vitamine B_{12}*, Val-Tyr*, pyrazine (99%), 2-methylpyrazine (99%) and sodium bromide (99%) were purchased from Sigma–Aldrich (Bornem). L–lysine (97%), arginine (98%), proline (99%), asparagine monohydrate (99%), 2,5-dimethylpyrazine (99%), 2-ethylpyrazine (98%), 2-ethyl-3-methylpyrazine (99%), 2-ethyl-3,5(6)-dimethylpyrazine (99.5%) and trichloroacetic acid (99%) were

purchased from Acros Organics, Thermo – Fisher Scientific (Erembodegem, Belgium). Aspartic acid (99%), DL–threonine (99%), L–tyrosine (99%) were purchased from Merck (Darmstadt, Germany). DL–serine, 2,3-dimethylpyrazine (99%) and 2,6-dimethylpyrazine (96%) were purchased from Janssen Chimica (Geel, Belgium). Magnesium chloride hexahydrate (99%), calcium carbonate (99%), potassium iodide (99.5%), sodium chloride (98%) and sea sand (acid washed and calcinated a.r.) were purchased from Chemlab Analytical (Zedelgem, Belgium). Whey protein isolate LACPRODAN DI-9224 was donated from Arla foods (Aarhus, Denmark).

* Reagents used as molecular weight markers.

2.2.2 Hydrolysis of whey protein

Whey protein isolate [84% protein, (N x 6.38)] was dissolved in potassium phosphate buffer 0.1M pH 7.8 at a concentration of 5.0 mg/ml, heated at 95 ^{o}C for 5 min and then cooled to room temperature. Trypsin powder was added at a ratio of 1:20 w:w (enzyme to protein), the resulting solution was incubated at 37 ^{o}C in a water bath during 20 h. Further, the solution was heated at 95 ^{o}C for 5 min to inactivate the enzyme and then frozen for posterior use. The free amino acid content of the hydrolyzed protein was analyzed and reported (**Table 5**). The non-hydrolyzed whey protein isolate did not contain free amino acids in detectable amounts. The protein hydrolysate was furthermore characterized with size exclusion chromatography as shown in **Figure 4A** and outlined below. Additional information regarding the total composition of the whey protein isolate can be found in the **Appendix 2**.

2.2.3 Size exclusion chromatography

Samples of dissolved whey protein and hydrolyzed whey protein were analyzed with an ÄKTA explorer LC coupled to a UV detector (GE Healthcare, Zaventem, Belgium) equipped with a Superdex Peptide 10/300 GL column (GE Healthcare, Zaventem). Column technical details: matrix - cross-linked agarose and dextran; average particle size - 13 µm; exclusion limit - 20000 Da; optimal separation range - 100 – 7000 Da; max sample volume - 500 µl; max peptide concentration – 10.0 mg/500 µl sample.

Working conditions: mobile phase - ammonium hydrogen carbonate buffer 0.15M pH 7.8; flow - 0.5 ml/min; total time (68 min); UV detection - 214 nm.

2.2.4 Free amino acid analysis

Whey protein isolate was dissolved in phosphate buffer 0.1M pH 7.8 at a concentration of 5.0 mg/ml, 1.0 ml of this solution and 1.0 ml of hydrolyzed whey protein solution (**Section 2.2.2**) were put in different micro-centrifuge tubes of 1.5 ml (Fisherbrand Fisher Scientific, Erembodegem, Belgium). Further, 0.4 ml of a 52.5 % (w:v) aqueous solution of trichloroacetic acid was added to each tube, reaching a final volume of 1.4 ml with a concentration of 15.0 % (w:v) trichloroacetic acid. The samples were cooled to 4.0 ^{o}C during 15 minutes and then centrifuged at 11750 g for ten minutes in a Spectrafuge 16 M centrifuge (Labnet International, NJ, USA). The samples were collected and filtered on a Millex LCR 0.45 µm low protein binding filter (Merck Millipore LTD, Cork, Ireland) and analyzed using HPLC with fluorescence detection.[145] Samples were analyzed in duplicate and the data values depicted in **Table 5** represent the mean value of these analysis.

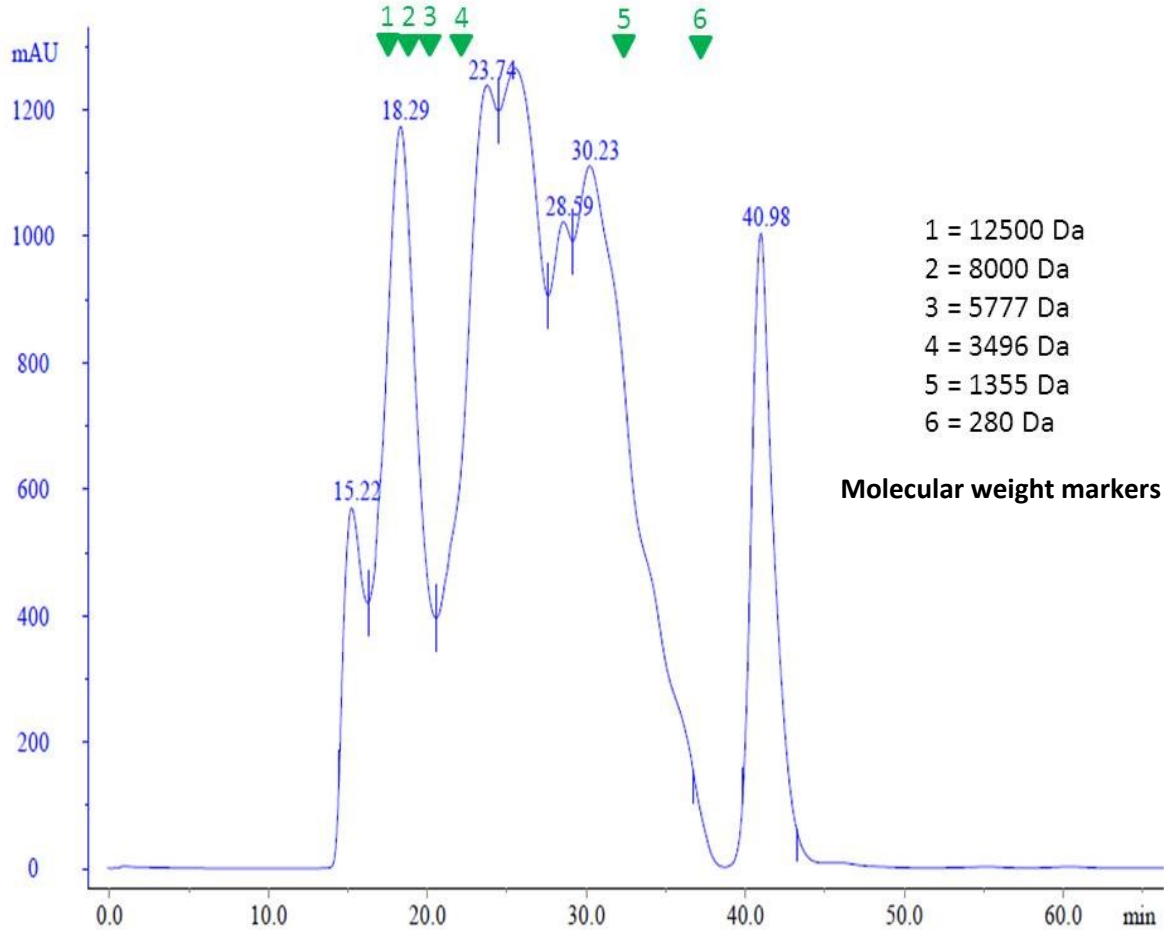


Figure 4A. Gel permeation chromatogram of hydrolyzed whey protein after tryptic hydrolysis. Non-hydrolyzed whey elutes as one unique peak at 15.22 minutes. Peak position for weight markers are shown on top

Table 5. Free amino acid composition of tryptic whey protein hydrolysate

Amino acid	mg/100 mg hydrolyzed whey
Lysine	0.9916
Leucine	0.1340
Valine	0.0960
Isoleucine	0.0728
Arginine	0.0546
Tyrosine	0.0421
Threonine	0.0389
Tryptophan	0.0359
Phenylalanine	0.0297
Methionine	0.0218
Alanine	0.0104

LOD: 10 nmols/ml

2.2.5 Selection of the experimental conditions

2.2.5.1 Reactant type and ratio

Two groups of samples were analyzed to evaluate the yields of pyrazines formation under roasting and sterilization conditions (see below).

Samples containing hydrolyzed whey protein and glucose were prepared at 8 different ratios (protein:glucose) (w:w) ranging from 1:0.08 to 1:1.33. The protein amount (15 mg) was kept constant while the amounts of glucose were added as indicated in **Table 8**. The quantities of hydrolyzed whey protein and glucose were respectively added from 5.0 mg/ml and 15.0 mg/ml solutions in potassium phosphate buffer 0.1M pH 7.8.

Samples containing lysine and glucose were prepared at 8 different ratios (lysine:glucose) (w:w) ranging from 1:0.15 to 1:2.53. The lysine amount (14.61 mg) was kept constant while the amounts of glucose were added as indicated in **Table 9**. The quantities of lysine and glucose were respectively added from 14.61 mg/ml and 18.0 mg/ml solutions in potassium phosphate buffer 0.1M pH 7.8.

All different samples were mixed in a volumetric flask and their final volume was adjusted to 5.0 ml.

2.2.5.2 Thermal conditions

2.2.5.2.1 Sterilization conditions

Previously obtained samples (5.0 ml) were transferred to a 20.0 ml flat bottom SPME vial (Gerstel, Mülheim, Germany) and closed with a pressure cap (silicon/PTFE 55^{o} shore A 1.5mm magnetic. Gerstel, Mülheim). The vial was heated in a stirring oil bath at 130 ^{o}C to obtain an inside temperature of 120 ^{o}C, for 2 h, after which the vials were immediately cooled down in an ice bath. Samples were prepared in triplicate.

2.2.5.2.2 Roasting conditions

Previously obtained samples (5.0 ml) were transferred to a 20.0 ml flat bottom SPME vial containing 1.5 g of sand. Subsequently, the samples were frozen and freeze-dried in a VaCo5 freeze-dryer (Zirbus Technology, Bad Grund, Germany) to ensure sample homogeneity. Finally, the samples were closed with pressure caps and heated in an oven (Memmert, Fisher Scientific, Erembodegem) at 180 ^{o}C for 90 min. Preliminary experiments showed that a considerable improvement of the reproducibility could be obtained by dissolving, freezing and freeze-drying instead of dry mixing the various ingredients. Samples were prepared in triplicate.

2.2.6 Elucidation of the role of protein hydrolysis products on the formation of pyrazines

Eight groups of model systems were prepared as follows:
(1) 15.0 mg of hydrolyzed whey protein and 15.0 mg of glucose, (2) 15.0 mg native whey protein and 15.0 mg glucose, (3) 15.0 mg native whey protein, 15.0 mg glucose and the addition of a mixture of free amino acids corresponding to the free amino acid content of the tryptic digestion of whey protein as reported in **Table 5**, (4) a mixture of free amino acids corresponding to the free amino acid content of the tryptic digestion of whey protein as reported in **Table 5** and 15.0 mg glucose, (5) 15.0 mg of hydrolyzed whey protein and 7.5 mg of glucose, (6) 15.0 mg native whey protein and 7.5 mg glucose, (7) 15.0 mg native whey protein, 7.5 mg glucose and the addition of a mixture of free amino acids corresponding to the free amino acid content of the tryptic digestion of whey protein as reported in **Table 5**, (8) a mixture of free amino acids corresponding to the free amino acid content of the tryptic digestion of whey protein as reported in **Table 5** and 7.5 mg glucose.

The quantities of native whey protein and hydrolyzed whey protein were respectively added from 5.0 mg/ml solutions in potassium phosphate buffer 0.1M pH 7.8. The quantities of glucose were taken from a 15.0 mg/ml solution in potassium phosphate buffer 0.1M pH 7.8.
The quantities of free amino acid were added from different solutions in potassium phosphate buffer 0.1M pH 7.8 as described in **Table 6**.

Table 6. Preparation of amino acid solutions in a concentration corresponding to their quantity in 15 mg of tryptic hydrolyzed whey protein

Amino acid	mg / 100 mg Hydrolyzed whey	mg / 15 mg Hydrolyzed whey	AA solutions made (mg/ml)	Volume added in the model systems (µl)
Lysine	0.9916	0.1487	7.4370	20
Leucine	0.1340	0.0201	1.0050	20
Valine	0.0960	0.0144	0.7200	20
Isoleucine	0.0728	0.0109	0.5460	20
Arginine	0.0546	0.0082	0.4095	20
Tyrosine	0.0421	0.0063	0.3158	20
Threonine	0.0389	0.0058	0.2918	20
Tryptophan	0.0359	0.0054	0.2693	20
Phenylalanine	0.0297	0.0045	0.2228	20
Methionine	0.0218	0.0033	0.1635	20
Alanine	0.0104	0.0016	0.0780	20

The quantities of all the different reagent solutions were mixed in a volumetric flask and their final volume was adjusted to 5.0 ml. The reaction mixtures were transferred to 20.0 ml SPME vials containing 1.5 g of sand, freeze-dried, closed with pressure caps and thermally treated under previously described roasting conditions.

2.2.7 Impact of a_w on pyrazine generation from hydrolyzed whey protein

Samples containing 15.0 mg of hydrolyzed whey protein and 7.5 mg of glucose taken from 5.0 mg/ml and 15.0 mg/ml solutions in potassium phosphate buffer 0.1M pH 7.8 were mixed in a volumetric flask and their final volume was adjusted to 5.0 ml. The reaction mixtures were transferred to 20.0 ml SPME vials containing 1.5 g of sand. Subsequently the samples were freeze-dried and the vials were immediately capped. Afterwards, the a_w value of the samples was determined using a 4TEV dew point water activity meter (Aqualab, Decagon Devices, Pullman, USA) obtaining an initial value of 0.16.

Subsequently, the freeze-dried samples were transferred to hermetic plastic recipients containing saturated solutions of different salts at 30 ^{o}C to reach specific relative humidities. Solutions were: magnesium chloride (32% RH), potassium carbonate (43% RH), sodium bromide (56% RH), potassium chloride (67% RH), sodium chloride (75% RH). All the samples were incubated in triplicates during 7 days at 30 ^{o}C inside an incubator (Memmert, Fisher Scientific, Erembodegem) in order to reach the desired a_w in each sample. After reaching equilibrium, all samples were thermally treated under previously described roasting conditions.

2.2.8 HS SPME – GCMS analysis

The volatiles produced during the different experimental conditions were extracted by means of headspace solid – phase microextraction (HS – SPME) for 30 minutes at 35.0 ^{o}C with a DVD/Car/PDMS fiber (Supelco, Bornem) with a multipurpose sampler (MPS – 2) (Gerstel). GC – MS analyses of the SPME extract were done with an Agilent 6890 GC Plus apparatus coupled to a quadrupole mass spectrometer 5973 MSD (Agilent Technologies, Diegem, Belgium) and equipped with a DB-5 capillary column (30.0 m length x 0.25 mm i.d; 0.25 µm film thickness) (Agilent Technologies). Working conditions were: transfer line to MSD 250 ^{o}C, carrier gas (He) 1.0 ml/min; ionization: EI 70eV; acquisition parameters: scanned m/z: 40-200 (2-10 min), 40-300 (10-20 min), 40-400 (>20 min); oven temperature started at 35 ^{o}C, held 5 min, programmed from 35 – 80 ^{o}C at 2 ^{o}C/min, held 2 min. Pyrazines were identified by comparison of the mass spectrum with mass spectral libraries (Nist 98, Wiley 6th and HPCH2205) and by comparison of the calculated linear retention indices with literature values. 2,5-Dimethylpyrazine and 2,6-dimethylpyrazine were reported together as 2,5(6)-dimethylpyrazine due to co-elution under the selected chromatographic conditions.

The generation of pyrazines was followed in a semiquantitative way by considering the absolute peak area of each individual pyrazine.[120,146-147] Although this approach does not allow absolute quantitation of each individual pyrazine, it is generally accepted as suitable for evaluating the pyrazine formation in a reliable way. For example, Yu et al.[147] studied some aroma compounds formed in Maillard model systems at different pH values using two different SPME fibers. The quantities were reported as the absolute peak areas of the compounds in the total ion chromatogram. Moreover, Adams et al.[146] reported volatile compounds generated upon heating of different melanoidins fractions, and expressed the quantities as a percentage of the total GC peak area.

2.2.9 Statistical analysis

All analyses were made using SPSS Statistics version 22 at a significance level of 95% ($p = 0.050$). Data points were normally distributed (Kolmogorov-Smirnov test: ($p < 0.050$) for all standardized residuals) and represent mean values of 3 independent determinations. Therefore, one way ANOVA was selected for statistical analysis. The Games – Howell correction was applied to control the family-wise error rate at 5% for all multiple pairwise comparisons.

2.3 RESULTS AND DISCUSSION

As mentioned in **Chapter 1**, the Maillard reaction generates hundreds of compounds in different foods. In Maillard model systems, the formation of volatile compounds is abundant and complex as well. Therefore, it is expected that the chromatographic analyses of the Maillard model systems report dozens of compounds (**Figure 5**). Indeed, besides pyrazines, some other volatiles were generated in the different model systems, for example, 3-methylbutanal, dimethyldisulfide, dimethyltrisulfide, benzaldehyde, 1-phenylethanone or acetylpyridine. Nevertheless, the objective of this study is to evaluate the formation of pyrazines in model systems under different conditions, therefore, pyrazines were the only volatiles reported.

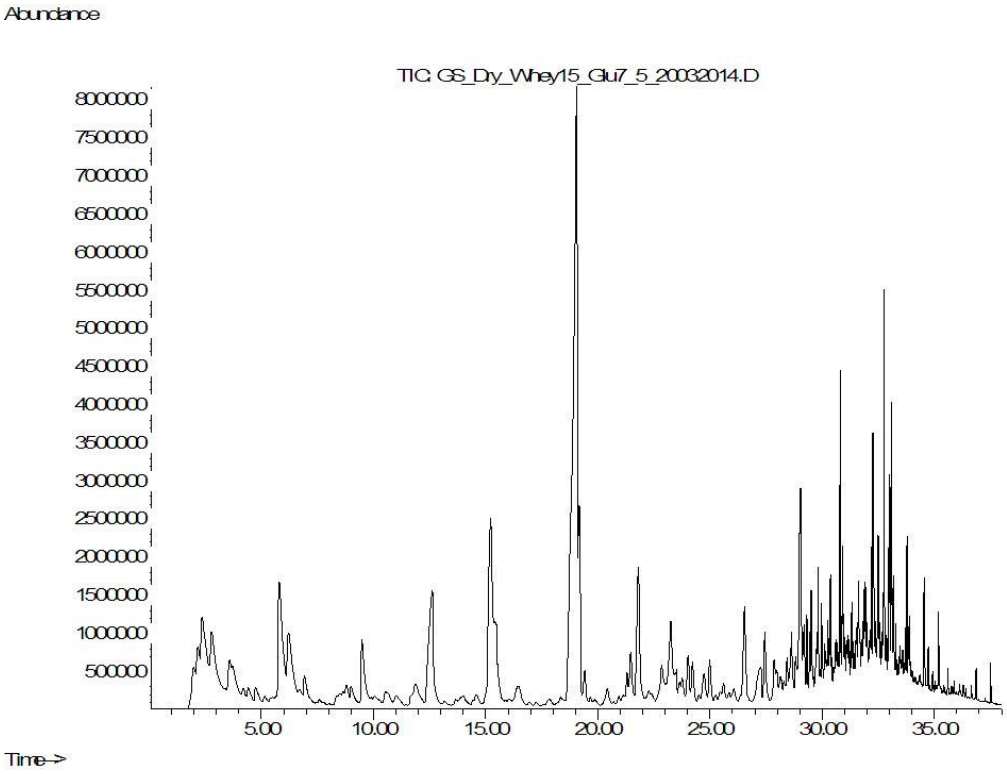


Figure 5. Full scan chromatogram of a Maillard model system between hydrolyzed whey and glucose in roasting conditions.

2.3.1 Selection of the reaction conditions and ratio of the model systems

In a first series of experiments, the impact of the reactant ratio (hydrolyzed whey protein and glucose) on the formation of pyrazines was evaluated for both thermal treatments applied. Several model systems containing peptides or proteins and glucose were reported in the literature. However, the information regarding how the authors selected their corresponding reactants ratio is limited. Lu et al.,[118] Oh et al.,[11,117] Kim et al.,[148] and Van Lancker et al.[120-121], used equimolar amounts of peptides and glucose for their model systems. However, Zhang et al.,[119] Lee et al.,[139] and Yang et al.[129] designed their respective model systems without specifying how the reactants ratio was selected. Since protein hydrolysates contain different peptides, equimolar reactant ratios could not be applied. Additionally, in related model systems like as reported by Zhang et al.,[119] there is not enough information with respect to the rationales the authors used to select their reactants ratio. Therefore, in the present set of experiments, the optimal ratio between hydrolyzed whey protein and glucose was determined experimentally ranging from 1:0.08 to 1:1.33 (w:w). The studied protein to glucose ratios can be related to some extent with various food products in which alkylpyrazines are often reported (**Table 7**).

Table 7. Protein to carbohydrate ratios of some food products

	Protein (g)	Total sugars (g)	Ratio protein to sugar
Almonds, flaked and ground [A]	21.1	4.2	1 : 0.20
Bread, wholemeal, average [A]	9.4	2.80	1 : 0.30
Cocoa powder [B]	18.5	1.75	1 : 0.09
Coffee beans (Robusta) [C]	15.0	12.5	1 : 0.83
Coffee beans (Arabica) [C]	11.0	11.50	1 : 1.05
Hazelnuts, kernel only [A]	14.1	4.00	1 : 0.28
Peanuts, dry roasted [A]	25.7	6.20	1 : 0.24
Pistachio nuts, kernel only, roasted and salted [A]	17.9	5.70	1 : 0.32
Potato chips, fine cut, from fast food outlets [A]	3.5	0.30	1 : 0.09

[A] Values obtained from The 'Composition of Foods Integrated Dataset' (CoFID), National Diet and Nutrition Survey, Public Health England (PHE). [B] Values obtained from the United States Department of Agriculture Agricultural Research Service National Nutrient Database for Standard Reference Release 28 . [C] Values obtained from Encyclopedia of Food and Health [149]

The results in **Table 8** indicate that during the roasting conditions (180 °C/90 min) significant quantities of the following pyrazines in model systems containing hydrolyzed whey protein and glucose are produced: 2-methylpyrazine, 2,5(6)-dimethylpyrazine, 2,3-dimethylpyrazine, 2-ethylpyrazine, 2-ethyl-3-methylpyrazine, 3-ethyl-2,5-dimethylpyrazine, 2-ethyl-3,5-dimethylpyrazine and 5-ethyl-2,3-dimethypyrazine.

The same reactants under sterilization conditions (120 °C/120 min) yielded mainly 2-methylpyrazine and 2,5(6)-dimethylpyrazine. However, the peak areas of the produced pyrazines were considerably lower than those observed during roasting conditions. These observations can be due to the fact that indeed a lower amount of pyrazines was produced, which can be explained by the different reaction conditions: temperature and presence of water in the systems. It should be noted as well, however, that by addition of 2.0 μg of 2-ethyl-3-methylpyrazine to similar amounts of non-thermally treated samples, a much higher peak area was observed for the dry (107.89×10^6) vs the wet sample (6.68×10^6). This indicates that the partitioning of the pyrazines between the samples and their headspace is quite different for a dry or aqueous sample.

The ratio between hydrolyzed whey protein and glucose played a crucial role in the formation of pyrazines. The generation of pyrazines under dry roasting conditions was low in model systems where glucose was present in low levels (ratio protein to glucose of 1:0.08 and 1:0.16). This effect can likely be explained since the amount of dicarbonyl compounds generated from glucose degradation was low, which had an impact on the formation of pyrazines. Higher peak areas were detected for ratios up to 1:0.5 and 1:0.67. At higher protein to glucose ratios, a decrease in the formation of pyrazines was again observed. This may be due to the excessive formation of different carboxylic acids such as acetic acid from glucose[150] which might decrease the pH of the model systems.

It is clear that the pH has an impact on the generation of pyrazines.[8] Their formation is hindered at low pH due to the protonation of the amino groups of the amino acids and peptides. Additionally, low pH affects directly the generation of pyrazines while favoring the 1,2-enolization mechanism of the Amadori or Heyns products, over the 2,3-enolization pathway in which the precursors for pyrazine generation are formed (**Section 1.4.2**). Also under sterilization conditions, the peak area of the formed pyrazines was affected by the protein/glucose ratio although to a lesser extent.

It is widely known that in Maillard reaction models free amino acids have a high reactivity. In order to compare the reactivity of the hydrolyzed whey protein/glucose with that of an amino acid/glucose mixture, lysine was selected. Pyrazine formation at different lysine/glucose ratios was monitored for both heating conditions (**Table 9**). Considerably higher levels of pyrazines were observed in the lysine containing reaction systems under both heating conditions when compared to hydrolyzed whey containing reaction systems. In addition, the detected pyrazines were more diverse, particularly for

the roasted samples. Under roasting conditions, the detected levels of 2-methylpyrazine and 2,5(6)-dimethylpyrazine were considerably less influenced by the lysine/glucose ratio. However, the lysine/glucose ratio did have a considerable effect on the amount of pyrazines detected. At the lower lysine/glucose ratios, formation of pyrazines in roasting conditions decreased considerably, while this tendency was not observed under sterilization conditions. These experiments show that depending upon the amino compounds participating in the reactions such as free amino acids or peptides, the impact of the amino compounds/glucose ratio on the pyrazine formation is different.

As it was noticed that considerable more pyrazines were detected under roasting conditions (180°C/90 min) either for hydrolyzed whey protein/glucose and lysine/glucose models, this heat treatment was further on used.

Table 8. Pyrazines (GC-MS peak Area x 10^{6}) detected in model reactions of dry glucose and hydrolyzed whey mixtures under roasting conditions (180 ^{0}C/90 min), and aqueous glucose and hydrolyzed whey solutions under sterilization conditions (120 ^{0}C/120 min).

Compound	Experimental conditions	Hydrolyzed whey 15.0 mg Glucose 1.25 mg	Hydrolyzed whey 15.0 mg Glucose 2.5 mg	Hydrolyzed whey 15.0 mg Glucose 5.0 mg	Hydrolyzed whey 15.0 mg Glucose 7.5 mg	Hydrolyzed whey 15.0 mg Glucose 10.0 mg	Hydrolyzed whey 15.0 mg Glucose 15.0 mg	Hydrolyzed whey 15.0 mg Glucose 17.5 mg	Hydrolyzed whey 15.0 mg Glucose 20.0 mg
2-Methylpyrazine [A]	Roasting	4.15 ± 0.12 a	9.46 ± 0.23 b	16.21 ± 0.47 c	23.10 ± 0.36 d	22.54 ± 1.95 cd	20.97 ± 1.20 cd	18.86 ± 1.47 cd	20.87 ± 0.65 d
	Sterilization	N/D	N/D	0.94 ± 0.04 a	1.75 ± 0.01 b	3.46 ± 0.24 c	5.45 ± 0.08 de	5.35 ± 0.29 de	6.32 ± 0.39 e
2,5(6)-Dimethylpyrazine [A]	Roasting	24.45 ± 1.35 a	35.22 ± 1.88 b	37.81 ± 1.23 bd	42.39 ± 3.04 d	35.25 ± 2.01 b	25.41 ± 1.83 a	22.75 ± 2.22 a	20.22 ± 1.26 a
	Sterilization	N/D	5.65 ± 0.52 a	6.75 ± 0.66 ac	9.64 ± 0.41 be	7.49 ± 0.19 c	11.16 ± 0.04 d	8.32 ± 0.20 e	9.66 ± 0.14 b
2,3-Dimethylpyrazine [A]	Roasting	N/D	N/D	1.13 ± 0.03 a	1.80 ± 0.07 b	2.10 ± 0.15 c	2.16 ± 0.01 c	2.54 ± 0.14 d	2.11 ± 0.03 c
	Sterilization	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
2-Ethylpyrazine [A]	Roasting	N/D	N/D	3.45 ± 0.04 a	4.24 ± 0.19 b	4.29 ± 0.16 b	4.73 ± 0.06 c	4.96 ± 0.22 c	4.92 ± 0.12 c
	Sterilization	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
2-Ethyl-3-methylpyrazine [A]	Roasting	3.79 ± 0.20 a	8.22 ± 0.44 bd	10.84 ± 0.21 c	11.00 ± 0.51 c	9.38 ± 0.48 d	8.80 ± 0.05 bd	9.32 ± 0.50 d	7.92 ± 0.37 b
	Sterilization	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
3-Ethyl-2,5-dimethylpyrazine [A]	Roasting	3.77 ± 0.18 a	6.70 ± 0.39 b	6.78 ± 0.33 b	5.14 ± 0.13 c	3.59 ± 0.33 a	3.37 ± 0.18 a	3.43 ± 0.21 a	2.42 ± 0.14 da
	Sterilization	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
2-Ethyl-3,5-dimethylpyrazine [A]	Roasting	N/D	N/D	2.07 ± 0.04 a	2.28 ± 0.14 a	2.04 ± 0.16 a	1.54 ± 0.08 ba	1.26 ± 0.56 b	1.27 ± 0.09 b
	Sterilization	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
5-Ethyl-2,3-dimethylpyrazine [B]	Roasting	N/D	N/D	1.22 ± 0.05 a	1.06 ± 0.06 a	1.03 ± 0.08 a	1.16 ± 0.08 a	1.23 ± 0.11 a	1.12 ± 0.03 a
	Sterilization	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
Ratio of hydrolyzed whey to glucose (w:w)		1:0.08	1:0.16	1:0.33	1:0.50	1:0.67	1:1.00	1:1.16	1:1.33

N/D (not detected). [A] Identification confirmed by GC retention index and mass spectra of authentic compounds. [B] Tentatively identified by matching mass spectra library.
Data points represent mean values of 3 independent determinations. Values in the same line followed by different letters are significantly different ($p < 0.05$).

Table 9. Pyrazines (GC-MS peak Area x 10^{6}) detected in model reactions of dry glucose and lysine mixtures under roasting conditions (180 ^{0}C/90 min), and aqueous glucose and lysine solutions under sterilization conditions (120 ^{0}C/120 min).

Compound	Experimental conditions	Lysine 14.61 mg Glucose 2.50 mg	Lysine 14.61 mg Glucose 4.50 mg	Lysine 14.61 mg Glucose 9.00 mg	Lysine 14.61 mg Glucose 13.5 mg	Lysine 14.61 mg Glucose 18.00 mg	Lysine 14.61 mg Glucose 22.50 mg	Lysine 14.61 mg Glucose 27.00 mg	Lysine 14.61 mg Glucose 31.50 mg	Lysine 14.61 mg Glucose 37.00 mg
2-Methylpyrazine [A]	Roasting	49.05 ± 0.11 a	36.80 ± 0.15 b	35.96 ± 0.83 b	42.74 ± 0.01 c	48.49 ± 0.87 a	45.16 ± 0.01 d	40.69 ± 1.13 e	43.36 ± 0.18 cd	40.57 ± 0.08 e
	Sterilization	N/D	6.75 ± 0.47 a	13.28 ± 0.19 b	15.11 ± 0.39 bc	16.97 ± 0.40 c	25.45 ± 1.15 d	30.33 ± 1.46 e	34.82 ± 2.04 f	38.87 ± 1.14 g
2,5(6)-Dimethylpyrazine [A]	Roasting	430.58 ± 0.01 a	426.55 ± 3.67 a	402.27 ± 1.03 b	450.48 ± 1.28 c	448.55 ± 0.51 c	433.84 ± 3.74 a	392.45 ± 9.19 b	293.06 ± 0.63 d	251.48 ± 1.27 e
	Sterilization	N/D	8.92 ± 0.77 a	19.07 ± 1.37 b	25.24 ± 0.59 dc	20.95 ± 1.74 b	27.52 ± 0.57 c	29.69 ± 1.33 c	25.57 ± 0.45 c	24.37 ± 1.14 d
2,3-Dimethylpyrazine [A]	Roasting	2.23 ± 0.03 a	4.12 ± 0.02 b	10.35 ± 0.22 c	13.81 ± 0.11 d	15.11 ± 0.13 e	14.62 ± 0.07 f	12.62 ± 0.27 g	10.62 ± 0.03 c	8.07 ± 0.05 h
	Sterilization	N/D	1.65 ± 0.16 a	2.39 ± 0.06 b	2.86 ± 0.17 bc	2.42 ± 0.03 b	3.17 ± 0.12 c	3.34 ± 0.29 c	3.41 ± 0.29 c	3.20 ± 0.23 c
2-Ethylpyrazine [A]	Roasting	N/D	N/D	8.37 ± 0.12 a	13.44 ± 0.16 b	15.37 ± 0.08 c	15.31 ± 0.33 c	11.81 ± 0.06 d	10.58 ± 0.06 e	8.88 ± 0.02 f
	Sterilization	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
2-Ethyl-3-methylpyrazine [A]	Roasting	45.50 ± 0.50 a	68.30 ± 0.16 b	211.13 ± 2.29 c	239.29 ± 0.69 d	278.17 ± 0.20 e	263.29 ± 1.66 f	182.96 ± 0.53 g	117.95 ± 2.19 h	75.22 ± 0.04 i
	Sterilization	N/D	N/D	N/D	1.10 ± 0.05 a	1.56 ± 0.08 b	2.00 ± 0.05 c	2.53 ± 0.05 d	2.75 ± 0.25 d	2.84 ± 0.08 d
3-Ethyl-2,5-dimethylpyrazine [A]	Roasting	47.89 ± 0.02 a	67.30 ± 0.57 b	89.62 ± 0.92 c	115.96 ± 2.02 d	121.61 ± 0.80 e	135.47 ± 1.47 f	91.24 ± 0.07 c	48.87 ± 0.56 a	26.95 ± 0.57 g
	Sterilization	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
2-Ethyl-3,5-dimethylpyrazine [A]	Roasting	4.80 ± 0.10 a	10.58 ± 0.20 b	32.99 ± 0.17 cd	31.58 ± 0.51 d	31.68 ± 0.32 d	32.41 ± 2.70 cd	35.29 ± 0.55 c	17.83 ± 0.43 e	9.96 ± 0.11 b
	Sterilization	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
5-Ethyl-2,3-dimethylpyrazine [B]	Roasting	7.86 ± 0.09 a	22.07 ± 0.20 b	101.59 ± 3.64 c	191.70 ± 5.51 d	222.19 ± 0.69 e	219.87 ± 6.20 e	85.99 ± 0.39 f	32.70 ± 0.01 b	13.72 ± 0.79 ab
	Sterilization	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
2,5-Diethylpyrazine [B]	Roasting	N/D	9.69 ± 0.09 a	56.94 ± 0.90 b	89.30 ± 1.28 c	94.04 ± 0.60 c	80.42 ± 4.16 d	23.77 ± 0.10 e	8.07 ± 0.58 a	2.70 ± 0.17 f
	Sterilization	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
2,3-Diethyl-5-methylpyrazine [B]	Roasting	N/D	3.11 ± 0.07 a	7.44 ± 0.07 b	11.18 ± 0.54 c	14.75 ± 0.01 d	16.65 ± 0.54 e	11.59 ± 0.29 c	5.59 ± 0.02 f	2.75 ± 0.07 a
	Sterilization	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
3,5-Diethyl-2-methylpyrazine [B]	Roasting	N/D	2.95 ± 0.09 a	10.30 ± 0.16 b	14.60 ± 0.32 c	18.20 ± 0.03 d	15.09 ± 0.75 c	8.40 ± 0.15 e	3.36 ± 0.13 a	1.37 ± 0.06 f
	Sterilization	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
2-Ethyl-3,5,6-trimethylpyrazine [B]	Roasting	N/D	3.29 ± 0.06 a	8.43 ± 0.16 b	13.05 ± 0.70 c	19.25 ± 0.11 d	16.99 ± 0.43 e	9.88 ± 0.14 f	4.50 ± 0.02 g	2.38 ± 0.23 a
	Sterilization	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
2-Isopropyl-3,5-dimethylpyrazine [B]	Roasting	N/D	11.11 ± 0.10 a	8.86 ± 0.06 b	9.38 ± 0.10 c	10.72 ± 0.19 d	12.41 ± 0.08 e	9.38 ± 0.11 c	6.59 ± 0.09 f	2.78 ± 0.11 g
	Sterilization	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
Ratio of lysine to glucose (w:w)		1:0.17	1:0.30	1:0.61	1:0.92	1:1.23	1:1.54	1:1.84	1:2.15	1:2.53

N/D (not detected). [A] Identification confirmed by GC retention index and mass spectra of authentic compounds. [B] Tentatively identified by matching mass spectra library. Data points represent mean values of 3 independent determinations. Values in the same line followed by different letters are significantly different ($p < 0.05$).

2.3.2 Elucidation of the role of protein hydrolysis products on the formation of pyrazines

From the previous experiments, it was obvious that the hydrolyzed whey proteins are able to generate pyrazines in Maillard reaction systems. However, it was not clear whether the compounds responsible for the pyrazine generation are the free amino acids produced during hydrolysis (**Table 5**), or the peptidic fraction, or both. Therefore, the formation of pyrazines by the hydrolyzed whey protein was compared with the pyrazine generation in a mixture of non-hydrolyzed proteins to which the free amino acids formed upon trypsinogenic hydrolysis were added according to the amounts reported in **Table 5**. Non-hydrolyzed whey protein was included as a control. In addition, glucose together with the free amino acids generated upon trypsinogenic hydrolysis of whey was considered as well. Experiments were conducted under dry heating conditions considering two protein/glucose ratios.

In model systems containing free amino acids in quantities as described in **Table 5** and glucose, no pyrazines were detected (data not shown). Most likely the amount of reagents was not sufficient to generate sufficient Maillard products. Model systems containing hydrolyzed whey protein and glucose led to the formation of the highest levels of pyrazines (**Table 10**). In model systems containing native whey protein and glucose, the generation of pyrazines was significantly lower than in samples of hydrolyzed whey. The native whey protein isolate did not contain any free amino acids, suggesting that the limited generation of pyrazines was due to thermal degradation of the protein in the selected reaction conditions, releasing amino compounds which can react with glucose and form pyrazines. Model systems containing native whey with the addition of the mixture of free amino acids (**Table 5**) and glucose produced comparable and statistically similar amounts of pyrazines as the model systems containing the native whey protein isolate and glucose.

These results allowed to conclude that the level of free amino acids present in the whey hydrolysate was not sufficient to produce pyrazines in measurable amounts using the heating conditions earlier described. Compared to the results from the experiments with the hydrolyzed whey, it is obvious that the peptides generated upon hydrolysis play an important role in the generation of pyrazines.

Regarding the pathways that involve the peptidic fraction into the generation of α-aminoketones and their posterior condensation into pyrazines, Oh et al.[11] studied model systems with glycine containing di-, tri- and tetrapeptides. The authors suggested that tetraglycine could be degraded to diglycine and further degraded into glycine to produce pyrazines. Simultaneously, triglycine was suggested to be degraded to diglycine and glycine. Yan et al.[129] studied peptide bond cleavage

during the Maillard reaction and their results show that some peptide bonds are more resistant or labile depending on the amino acid sequence in the peptide chain. The mechanism previously depicted in **Scheme 10** (**Section 1.7.3**) implies the formation of pyrazines in model systems containing dipeptides and dicarbonyl compounds. The alternative mechanism was suggested since dipeptides cannot follow the typical Strecker degradation due the absence of a free carboxyl group to form α-aminoketones.

It is obvious that a similar mechanism can be responsible for the formation of pyrazine from oligopeptides as well.

2.3.3 Impact of a_w on pyrazine generation from hydrolyzed whey protein

The water content of the samples was controlled by equilibrating them in recipients filled with saturated salt solutions, obtaining atmospheres with fixed relative humidities as described by Greenspan et al.[151] Thus, after equilibration, samples with a particular a_w were obtained.

The results presented in **Table 11** show that the model systems incubated with a a_w of 0.33 are the ones that produced the highest amounts and variety of pyrazines. This may be explained by the potential effect of a_w on the peptide degradation. Oliyai et al.[152] studied the chemical stability of a hexapeptide under several factors such as pH, temperature and moisture content, finding that the moisture content degraded significantly the hexapeptide, leading to an increased reactivity. In addition, a_w has been pointed out to have an impact on reaction rates[153] since water in food often acts as a plasticizer, leading to enhanced mobility and chemical reactivity as well.[154] However, increasing the a_w to values higher than 0.33 leads progressively to a lower generation of pyrazines, which can be explained by the fact that water is a product of several condensation steps in the Maillard reaction.[155]

Table 10. Pyrazines (GC-MS peak Area x 10^{6}) detected in model reactions of dry mixtures of glucose and hydrolyzed whey, whey and whey with the addition of an amount of free amino acids as described in **Table 5**, under roasting conditions (180 ^{0}C/90 min)

Compound	Hydrolyzed whey 15.0 mg Glucose 7.5 mg	Hydrolyzed whey 15.0 mg Glucose 15.0 mg	Whey 15.0 mg Glucose 7.5 mg	Whey 15.0 mg Glucose 15.0 mg	Whey 15.0 mg + AA (*) Glucose 7.5 mg	Whey 15.0 mg + AA (*) Glucose 15 mg
2-Methylpyrazine [A]	23.10 ± 0.36 a	20.97 ± 1.20 b	8.99 ± 0.62 c	8.53 ± 0.26 c	9.66 ± 1.05 d	8.06 ± 0.66 e
2,5(6)-Dimethylpyrazine [A]	42.39 ± 3.04 a	25.41 ± 1.83 b	9.02 ± 0.69 c	9.29 ± 0.82 c	10.99 ± 1.17 d	9.37 ± 0.84 c
2,3-Dimethylpyrazine [A]	1.80 ± 0.07 a	2.17 ± 0.01 a	N/D	N/D	N/D	N/D
2-Ethylpyrazine [A]	4.23 ± 0.19 a	4.73 ± 0.06 a	N/D	N/D	N/D	N/D
2-Ethyl-3-methylpyrazine [A]	10.70 ± 0.11 a	8.81 ± 0.05 b	1.64 ± 0.05 c	3.50 ± 0.13 d	3.53 ± 0.12 d	3.44 ± 0.42 d
3-Ethyl-2,5-dimethylpyrazine [A]	5.14 ± 0.13 a	3.38 ± 0.18 b	N/D	N/D	N/D	N/D
2-Ethyl-3,5-dimethylpyrazine [B]	2.28 ± 0.14 a	1.54 ± 0.08 b	N/D	N/D	N/D	N/D
5-Ethyl-2,3-dimethylpyrazine [B]	1.03 ± 0.01 a	1.16 ± 0.08 a	N/D	N/D	N/D	N/D
Ratio of protein to glucose (w:w)	1:0.50	1:1.00	1:0.50	1:1.00	1:0.50	1:1.00

N/D (not detected). (*) concentration of amino acids as described in **Table 5**. [A] Identification confirmed by GC retention index and mass spectra of authentic compounds. [B] Tentatively identified by matching mass spectra library. Data points represent mean values of 3 independent determinations. Values in the same line followed by different superscript letters are significantly different ($p < 0.05$).

Table 11. Pyrazines (GC-MS peak Area x 10^{6}) detected in model reactions of dry glucose and hydrolyzed whey mixtures incubated at different relative humidities under roasting conditions (180 ^{0}C/90 min)

Compound	a_w 0.16	a_w 0.33	a_w 0.38	a_w 0.53	a_w 0.62	a_w 0.75
2-Methylpyrazine [A]	23.10 ± 0.36 a	54.45 ± 4.70 b	37.99 ± 1.23 c	30.40 ± 1.25 d	22.39 ± 0.86 a	19.71 ± 0.89 a
2,5(6)-Dimethylpyrazine [A]	42.39 ± 3.04 a	419.99 ± 13.07 b	343.52 ± 16.20 c	327.52 ± 14.63 c	287.13 ± 5.82 d	211.5 ± 12.18 e
2,3-Dimethylpyrazine [A]	1.8 ± 0.07 a	5.43 ± 0.26 b	5.73 ± 0.54 b	5.02 ± 0.48 b	4.78 ± 0.37 b	4.59 ± 0.33 b
2-Ethylpyrazine [A]	4.24 ± 0.19 a	6.69 ± 0.46 b	5.62 ± 0.40 b	4.84 ± 0.43 ca	5.07 ± 0.28 ca	4.98 ± 0.32 ca
2-Ethyl-3-methylpyrazine [A]	11.00 ± 0.51 a	122.23 ± 8.24 b	101.74 ± 6.33 b	97.00 ± 6.95 cd	107.68 ± 6.33 bc	85.2 ± 8.00 d
3-Ethyl-2,5-dimethylpyrazine [A]	5.14 ± 0.13 a	262.84 ± 12.30 b	249.39 ± 13.11 b	247.43 ± 12.75 b	254.57 ± 4.56 b	163.00 ± 11.38 c
2-Ethyl-3,5-dimethylpyrazine [A]	2.28 ± 0.14 a	30.34 ± 2.08 b	24.82 ± 1.93 c	22.04 ± 2.05 cd	22.26 ± 1.37 c	16.61 ± 1.39 d
5-Ethyl-2,3-dimethylpyrazine [B]	1.06 ± 0.06 a	28.34 ± 2.46 b	19.73 ± 1.92 c	22.68 ± 1.15 c	30.82 ± 2.75 b	22.60 ± 1.67 c
2,5-Diethylpyrazine [B]	N/D	6.78 ± 0.33 a	5.10 ± 0.43 a	5.09 ± 0.45 a	9.92 ± 0.77 b	7.43 ± 0.70 ca
2,3-Diethyl-5-methylpyrazine [B]	N/D	18.72 ± 0.49 a	16.19 ± 1.05 ab	15.20 ± 0.46 b	18.59 ± 1.21 a	13.49 ± 1.27 b
3,5-Diethyl-2-methylpyrazine [B]	N/D	20.44 ± 0.37 a	18.07 ± 1.33 a	19.14 ± 1.28 a	26.53 ± 1.77 b	20.04 ± 1.93 a
2-Ethyl-3,5,6-trimethylpyrazine [B]	N/D	13.03 ± 0.93 a	11.46 ± 0.84 a	10.60 ± 0.72 ab	12.83 ± 1.02 a	8.81 ± 0.65 b
2,5-Dimethyl-3-(3-methyl-butyl)pyrazine [B]	N/D	47.25 ± 2.70 a	46.88 ± 1.58 a	43.83 ± 1.14 a	42.22 ± 4.01 a	22.55 ± 1.39 b
3-Furan-2-ylmethyl-2,5-dimethylpyrazine [B]	N/D	11.80 ± 0.95 a	0.39 ± 0.03 b	0.10 ± 0.01 b	0.07 ± 0.01 b	N/D

N/D (not detected). [A] Identification confirmed by GC retention index and mass spectra of authentic compounds. [B] Tentatively identified by matching mass spectra library.

Data points represent mean values of 3 independent determinations. Values in the same line followed by different superscript letters are significantly different ($p < 0.05$).

2.4 CONCLUSIONS

The generation of pyrazines was clearly enhanced during roasting conditions, while the ratio of the reactants also played an important role. Additionally, the production of pyrazines was favored at a a_w of 0.33 while it decreased at higher values. Nevertheless, the clearest conclusion was that the results suggest that the contribution of peptides to the generation of pyrazines is considerably high, while the role of free amino acids is only minor and more than likely less important.

As it was revealed that in a trypsinogenic hydrolysate of whey protein, peptides contributed significantly to the generation of pyrazines upon heating in the presence of glucose, the following chapter investigates the potential contribution of specific peptide fractions. The models were made using the protein to glucose ratio, a_w and heating conditions that were found to be the most suitable in the present chapter.

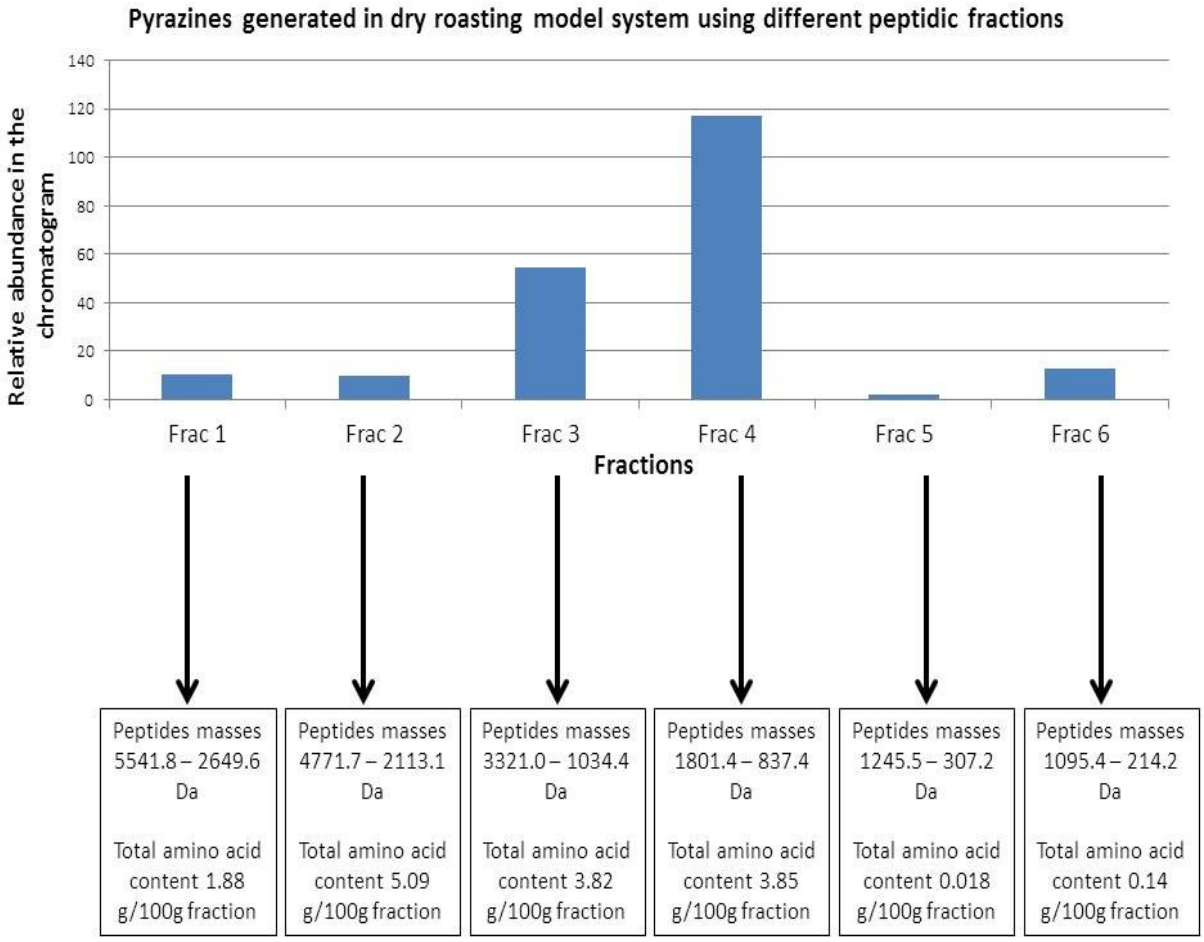
The role of peptides in food in the generation of pyrazines has probably been underestimated and requires more attention.

CHAPTER 3

The role of specific peptide fractions of tryptic hydrolyzed whey in the generation of alkylpyrazines in Maillard model systems

ABSTRACT

Tryptic whey protein hydrolysate was reported to be able to generate pyrazines in Maillard model systems, specially due to the presence of peptides. However, the role of particular peptide fractions in this observed phenomenon is unclear. The hydrolysate was divided in six fractions obtained via preparative gel permeation chromatography which are further evaluated for their capacity to generate pyrazines. It was found that all the fractions are able to generate pyrazines in the Maillard model systems. However, pyrazines were produced in higher amounts in fractions of low molecular weight. Moreover, the results suggest that the peptide sequence and the *N*-terminal amino acid of the various peptides did not influence the generation of pyrazines in the considered protein hydrolysate.



Graphic Abstract 2. Summarized information of **Chapter 3**

3.1 INTRODUCTION

Protein hydrolysates and oligopeptides have been reported previously as effective pyrazine precursors during the Maillard reaction. Nevertheless, to the best of our knowledge, little information is available regarding which are the most reactive peptides, or what is their optimal size. As it was mentioned in **Section 1.7.3** the formation of pyrazines involves the reaction of dicarbonyl compounds with the *N*-terminal amino acid of a peptide. Van Lancker et al. reported that the *N*-terminal amino acid has a significant influence on the formation of pyrazines in peptide containing model systems.[121] For example, it was noted by the authors that when leucine and valine were the *N*-terminal amino acids of several dipeptides, the production of pyrazines was quite similar and proceeded slower than with other dipeptides. At the same time, model systems of dipeptides, which contained lysine as the *N*-terminal amino acid also generated pyrazines in a similar way, without observing much influence of neighbor amino acids of the dipeptide.[120]

Therefore, since a tryptic whey protein hydrolysate was effective to generate pyrazines as reported in **Chapter 2**, the current study aimed to isolate different peptidic fractions from this hydrolysate and characterize them. The main objective was to evaluate the reactivity of the different fractions in Maillard model systems with respect to pyrazine formation.

3.2 MATERIALS AND METHODS

3.2.1 Samples and Chemicals

DL–norvaline (99%), L–cysteine (99.5%), L–4-hydroxyproline (99%), DL–valine (99%), DL–alanine (99%), L–tryptophan (99.5%), L–citruline (99%), sarcosine (99%), DL–histidine (99%), L–isoleucine (99.5%), DL–leucine (99%) and glutamine (99,5%) were purchased from Fluka (Sigma–Aldrich, Bornem, Belgium). DL–lysine monohydrochloride (98%), DL–methionine (99%), glycine (99.5%), L–arginine hydrochloride (99%), L–phenylalanine (99%), L–glutamic acid (99%), D-(+)-glucose (99.5%), trypsin from porcine pancreas, trypsin-chymotrypsin inhibitor from glycine max (soybean)*, insulin from bovine pancreas*, insulin chain B oxidized from bovine pancreas*, cytochrome C from equine heart*, vitamine B_{12}*, Val-Tyr*, pyrazine (99%), 2-methylpyrazine (99%) ammonium bicarbonate (99%) α-cyano-4-hydroxycinnamic acid (99%) and ammonium citrate dibasic (98%) were purchased from Sigma–Aldrich (Bornem). L–lysine (97%), arginine (98%), proline (99%), asparagine monohydrate (99%), 2,5-dimethylpyrazine (99%), 2-ethyl-3-methylpyrazine (99%) and 2-ethyl-3,5(6)-dimethylpyrazine (99.5%) were purchased from Acros Organics, Thermo – Fisher Scientific (Erembodegem, Belgium). Trifluoroacetic acid (Sequencer Grade) was purchased from Life

technologies Europe, Thermo – Fisher Scientific (Erembodegem, Belgium). Aspartic acid (99%), DL–threonine (99%), L–tyrosine (99%) were purchased from Merck (Darmstadt, Germany). DL–serine, 2,3-dimethylpyrazine (99%) and 2,6-dimethylpyrazine (96%) were purchased from Janssen Chimica (Geel, Belgium). Magnesium chloride hexahydrate (99%) and sea sand (acid washed and calcinated a.r.) were purchased from Chemlab Analytical (Zedelgem, Belgium). Acetonitrile HPLC-S (99.95%) was purchased from Biosolve (Valkenswaard, The Netherlands). Whey protein isolate LACPRODAN DI-9224 was donated from Arla foods (Aarhus, Denmark).

* Reagents used as molecular weight markers.

3.2.2 Hydrolysis of whey protein

The hydrolysis of the whey protein isolate was achieved by following the procedure described in **Section 2.2.2**.

3.2.3 Fractionation by preparative size exclusion chromatography

Samples of tryptic hydrolyzed whey protein were fractionated with an ÄKTA explorer LC coupled to a UV detector (GE Healthcare, Zaventem, Belgium) equipped with a Superdex Peptide 10/300 GL column (GE Healthcare, Zaventem). Column technical details: (see **Section 2.2.3**).

The software used to control the ÄKTA explorer expresses time units using a decimal system. Therefore, six fractions were collected at the following retention times: F1 (14.07 – 16.63 min), F2 (16.83 – 20.43 min), F3 (20.61 – 27.73 min), F4 (27.98 – 35.06), F5 (35.23 – 38.52 min), F6 (38.74 – 43.20 min). The fractionation step was repeated several times in order to obtain sufficient amount of peptides. Moreover, for each fractionation, the individual fractions were collected in a recipient and were frozen. The collected fractions were freeze-dried in a VaCo5 freeze-dryer (Zirbus Technology, Bad Grund, Germany) and posteriorly desalted using membrane dialysis against demineralized water using a Biotech CE Dialysis Tubing, 0.1-0.5 kD MWCO (VWR International, Leuven, Belgium). The dialysis membranes were placed in 5.0 l buckets containing demineralized water, the system was constantly stirred and the water was changed seven times in a period of one hour per change. After the last change the dialysis membranes were left on water overnight. After dialysis, the fractions were freeze-dried, re-dissolved in 0.1M potassium phosphate buffer pH 10, freeze-dried once again and stored for further use. This last step was repeated five times due to a persistent ammonia residue, for each repetition, new 0.1M potassium phosphate buffer pH 10 was added.

3.2.4 Total amino acid profile

The different fractions obtained from the hydrolysis of whey protein were hydrolyzed to their corresponding amino acids and further analyzed using HPLC – analysis with fluorescence detection as described by Mestdagh et al.[145]

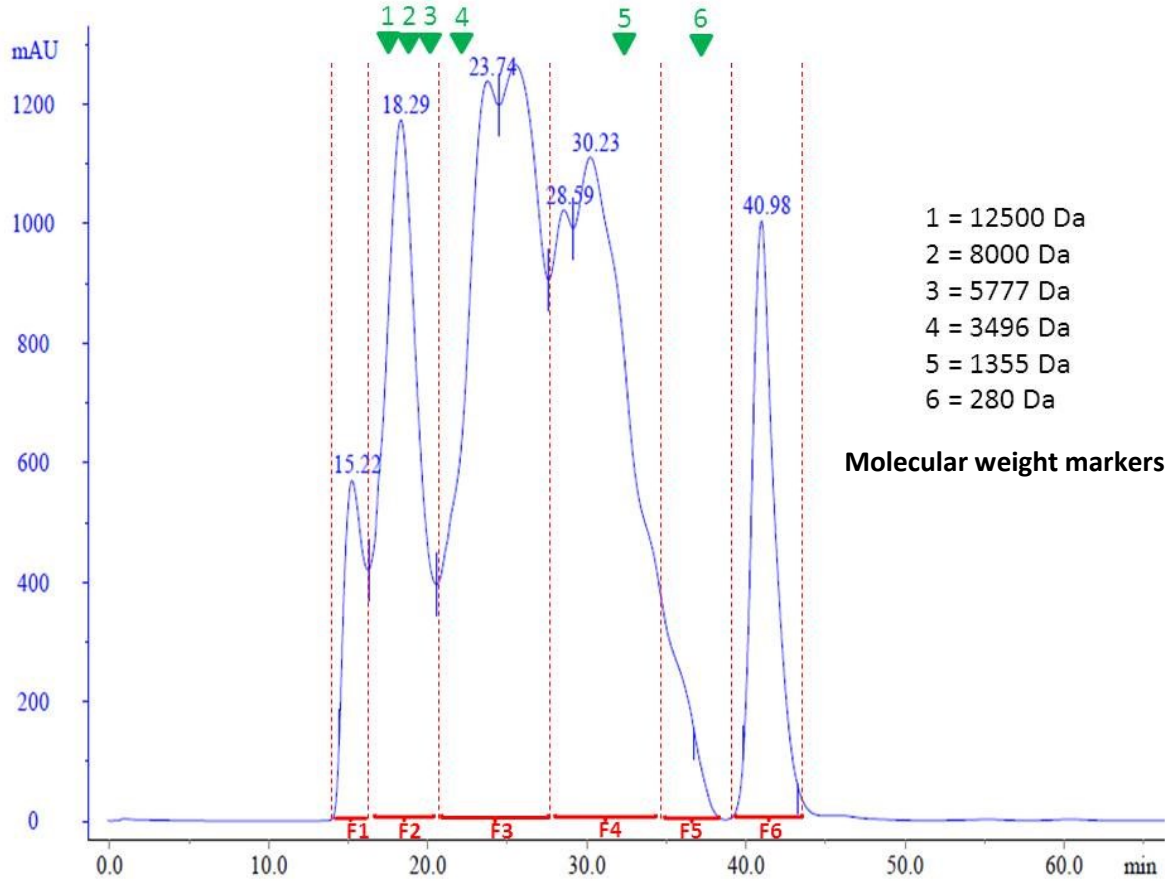


Figure 4B. Gel permeation chromatogram of hydrolyzed whey protein after tryptic hydrolysis. Peak position for weight markers are shown on top. F1 – F6 correspond to the retention time intervals for which the different fractions were collected.

3.2.5 MALDI-TOF analysis

MALDI – TOF – MS and MALDI – TOF – Tandem MS

Prior to spotting 1.0 µl of a 1/1 sample/matrix mix on an Optitof 384 well MALDI target plate, (ABSCIEX, Framingham, MA 01701, USA), the fractions were desalted and concentrated using ZipTip C_{18} or C_4 Millipore (Merck Millipore, Darmstadt, Germany), depending on their estimated mass range. α-Cyano-4-hydroxycinnamic acid was used as the matrix in a solution containing of 5.0 mg in 50% acetonitrile/10.0 mM citric acid/0.1% trifluoroacetic acid.

MS and tandem MS spectra were acquired on a 4800 Proteomics Analyzer, a MALDI-TOF-TOF instrument (ABSCIEX, Framingham, MA 01701, USA), using the delayed extraction technology in the positive reflector and linear mode.

Data analyses were obtained using 4000 Series Explorer and Data explorer software.

Protein identification was obtained by applying the Mascot Search Engine against the Swissprot and NCBInr databases, taxonomy Mammalia and implying decoy database searches. The search parameters were set to monoisotopic mass values with a peptide charge of +1 and the peptide mass tolerance was set to 200ppm, with a maximum of missed tryptic cleavages of 2. Methionine oxidation was set as a variable modification and the significance tolerance threshold was set below 0.05.

3.2.6 Pyrazine formation in Maillard model systems of the peptidic fractions and glucose

Previous experiments have shown that in model systems the optimal protein to glucose ratio was 1:0.50 (w/w) (**Section 2.3.1**). Therefore, this ratio was selected for this set of experiments supposing that the freeze-dried consist in pure protein/peptides.

The different fractions were dissolved separately in 0.1M potassium phosphate buffer pH 7.8 at a concentration of 5.0 mg of dry powder per ml of buffer. For each fraction, 3.0 ml of its corresponding solution were added to a volumetric flask together with 0.5 ml of a glucose solution in phosphate buffer at a concentration of 15.0 mg/ml. The volume of the mixture was adjusted to 5.0 ml with 0.1M potassium phosphate buffer pH 7.8. The mixture was transferred to a 20.0 ml SPME vial containing 1.5 g of sand and was frozen and freeze-dried in a VaCo5 freeze-dryer (Zirbus Technology, Bad Grund, Germany) to ensure sample homogeneity (**Section 2.2.5.2.2**).

The freeze-dried samples were subsequently transferred to a hermetic plastic recipient containing a saturated solution of magnesium chloride (32% RH) and incubated during four days at 30.0 ^{o}C in an incubator (Memmert, Fisher Scientific, Erembodegem, Belgium) in order to reach a stable a_w in each sample. After reaching equilibrium, the recipients were closed with pressure caps and heated in an oven (Memmert, Fisher Scientific) at 180 ^{o}C for 90 min.

The samples were analyzed by headspace coupled with gas chromatography/mass spectrometry (HS–GC/MS).

3.2.7 GC-MS conditions

The volatiles produced during the different experimental conditions were extracted by means of headspace solid – phase microextraction (HS – SPME) for 30 minutes at 35.0 ^{o}C with a DVD/Car/PDMS fiber (Supelco, Bornem) with a multipurpose sampler (CombiPAL), (CTC Analitics, Agilent technologies). GC – MS analyses of the SPME extract were obtained with an Agilent 7890A GC Plus apparatus coupled to a quadrupole mass spectrometer 5975 MSD (Agilent Technologies, Diegem, Belgium) and equipped with an DB-624 capillary column (60 m length x 0.250 µm i.d; 1.4 µm film thickness) (Agilent Technologies, Diegem). Working conditions were: transfer line to MSD 280 ^{o}C, carrier gas (He) 1.0 ml/min; ionization: EI 70eV; acquisition parameters: scanned m/z: 40-200 (2-10 min), 40-300 (10-20 min), 40-400 (>20 min); oven temperature started at 35 ^{o}C, held for 1 min, programmed from 35 – 120 ^{o}C at 15 ^{o}C/min, then from 120 – 130 ^{o}C at 2 ^{o}C/min and held for 2 min, then from 130 – 150 ^{o}C at 2 ^{o}C/min and from 150 – 250 ^{o}C at 20 ^{o}C/min and held 5 min. 2-methylpyrazine, 2,5-dimethylpyrazine, 2-ethyl-3-methylpyrazine and 2-ethyl-3,5(6)-dimethylpyrazine were identified by comparison of the mass spectrum and retention time of the authentic compounds.

The generation of pyrazines was followed in a semiquantitative way by considering the absolute peak area of each individual pyrazine (**Section 2.2.8**).

3.2.8 Statistical analysis

All analyses were made using SPSS Statistics version 22 at a significance level of 95% ($p = 0.050$). Data were normally distributed (Kolmogorov-Smirnov test: $p < 0.050$ for all standardized residuals) and represent mean values of 3 independent determinations. Therefore, one way ANOVA was selected for statistical analysis. The Games – Howell correction was applied to control the family-wise error rate at 5% for all multiple pairwise comparisons.

3.3 RESULTS AND DISCUSSION

3.3.1 Fractionation and characterization of tryptic whey protein hydrolysate

In the previous chapter, it was demonstrated that the tryptic whey protein hydrolysate is capable of generating alkylpyrazines in a variety of conditions. It was shown that the peptides present in this hydrolysate had a major role in the generation of alkylpyrazines while the free amino acids present in

the hydrolysate had a minor impact. Therefore, it was intended to determine which fractions from the tryptic hydrolysate were most reactive.

The fractionation of the hydrolysate was achieved using preparative GPLC, in order to obtain peptidic fractions in sufficient quantities to perform further experiments, more specifically related to their potential to produce alkylpyrazines.

As shown in **Figure 4B**, the separation of the peptidic fractions proved to be quite difficult due to a considerable overlapping of the peaks. This is not surprising as preparative chromatography has a low resolution. It was decided to collect six fractions based on their estimated molecular weight. Moreover, the individual fractions obtained after several GLPC fractionations were collected in the same recipient in order to be freeze-dried more effectively. Since the fractionation of the samples implied several collections and freeze-drying steps, the use of sodium or potassium phosphate buffer was not suitable due to accumulation of salts. Therefore, ammonium bicarbonate buffer was used due to its volatility. However, after freeze-drying, a strong ammonia odor was present in the dry fractions. It has been described in the literature review that ammonia is a pyrazine precursor in the presence of sugars (**Section 1.7**). Therefore, in order to avoid interference on the experimental design, ammonia was eliminated from the fractions membrane dialysis and freeze-drying (**Section 3.2.3**). After dialysis, the fractions were freeze-dried, re-dissolved in 0.1M potassium phosphate buffer pH 10, freeze-dried once again until the ammonia residue was eliminated. Due to this last step (**Section 3.2.3**), an increased concentration of potassium phosphate was expected in the dry fractions. The different fractions were used in Maillard model systems to evaluate the generation of pyrazines prior to characterize them by MALDI-TOF-MS and MALDI-TOF-Tandem MS analyses. It was found that the generated pyrazine levels were lower as in previous experiments. Therefore, a total amino acid analysis was done in order to estimate the protein content of each fraction.

As shown in **Table 12**, the total amount of amino acids present in all the fractions was very low, especially in fractions 5 and 6. Therefore, the protein to glucose ratios of the Maillard model systems was variable. Unfortunately, these experiments could not be repeated due to the limited amount of remaining sample.

3.3.2 MALDI-TOF-MS and MALDI-TOF-Tandem MS analysis

Each of the fractions was analyzed using MALDI-TOF-MS and MALDI-TOF-Tandem MS to determine the masses and, if possible, the sequences of the peptides present in the fractions. As the fractions contained only a small quantity of protein, all the different fractions had to be desalted prior to their analysis. Therefore, each fraction was desalted using C_{18} or C_4 ZipTips (Merck Millipore) depending on the estimated molecular weight of the fractions.

Table 12. Total amino acid profile of fractions 1 – 6 (Frac 1 – 6).

Compound	Frac 1	Frac 2	Frac 3	Frac 4	Frac 5	Frac 6
	mg/100mg					
Aspartate	0.139	0.315	0.453	0.566	N/D	N/D
Glutamate	0.441	1.337	1.187	0.692	N/D	N/D
Serine	0.103	0.338	0.145	0.064	N/D	N/D
Histidine	N/D	N/D	0.063	0.056	N/D	N/D
Glycine	0.045	0.094	0.210	0.073	0.018	0.033
Threonine	0.057	0.760	0.044	0.116	N/D	N/D
Arginine	0.069	0.047	0.111	0.078	N/D	N/D
Alanine	0.095	0.286	0.090	0.257	N/D	0.027
Tyrosine	0.111	0.056	0.200	0.040	N/D	0.044
Valine	0.135	0.438	0.055	0.076	N/D	0.027
Methionine	0.079	0.040	0.019	0.052	N/D	N/D
Phenylalanine	0.080	0.054	0.108	0.080	N/D	N/D
Isoleucine	0.078	0.444	0.212	0.406	N/D	N/D
Leucine	0.291	0.243	0.437	0.398	N/D	0.024
Lysine	0.102	0.068	0.212	0.647	N/D	N/D
Proline	0.055	0.564	0.271	0.254	N/D	N/D
SUM	1.879	5.085	3.817	3.854	0.018	0.154

The results in **Table 13** illustrate that the peptides with the heavier masses were mainly present in the fractions 1 and 2, lighter masses are found in fractions 3 – 5, and the lowest in fraction 6. These results are in line to what is shown in the chromatogram of the GPLC profile of the whey protein hydrolysate (**Figure 4B**). However, some fractions exhibited a wide range of masses due to the low resolution of the separation technique.

As the protein content of the fractions was quite low, the MALDI-MS-MS analysis was not possible for all the detected peaks. Therefore, the identification of the sequence of the peptides was rather difficult. Only three peptides could be identified by MS-MS spectra matching the results of the database. Several other peptide sequences were tentatively identified (**Table 14**) based on the mass of the peptides generated upon the tryptic hydrolysis of β-lactoglobulin and α-lactalbumin, which are the two main components of whey protein isolate. This comparison was made using the Uniprot database.[156]

As shown in **Table 14**, the peptides that were identified by MS-MS and the ones tentatively identified possess a considerable variety of *N*-terminal amino acids. Van Lancker et al. reported that the *N*-terminal amino acid has a significant influence on the formation of pyrazines in peptide containing model systems.[121]

Table 13. List of masses detected by MALDI-TOF-MS in the peptidic fractions of tryptic hydrolyzed whey

Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5	Fraction 6
Mass (m/z) of the compounds is given in Daltons (Da)					
5541.81	4771.71	3321.03	1801.42	1245.52	1095.48
5353.62	4740.13	3304.71	1770.50	1014.54	1034.49
5325.71	4653.55	3277.30	1772.44	955.44	955.37
5298.10	4284.31	3175.61	1755.42	953.45	903.41
4284.31	4279.94	3146.40	1746.42	952.46	841.32
3444.14	4251.39	2783.82	1715.44	949.45	751.32
3368.16	3800.53	2778.11	1684.49	944.45	696.27
2710.88	3368.14	2765.60	1668.43	939.45	654.33
2679.01	3326.26	2707.09	1642.44	937.43	341.24
2649.60	2710.87	2694.60	1340.47	936.47	313.20
	2678.11	2647.14	1303.38	933.46	307.15
	2649.62	2459.20	1252.38	673.31	246.00
	2315.54	2315.50	1199.37	674.40	244.19
	2141.70	2286.80	1196.44	667.32	214.18
	2113.06	2211.10	1147.32	650.33	
		2147.20	1140.38	573.34	
		2111.50	1127.36	549.26	
		2091.81	1122.27	546.39	
		2063.50	1100.38	325.21	
		2030.00	1064.40	307.20	
		1963.84	1065.58		
		1943.90	1058.32		
		1658.70	1034.32		
		1642.67	999.31		
		1627.80	977.37		
		1245.52	951.36		
		1065.38	949.37		
		1064.39	935.38		
		1057.50	933.39		
		1034.45	916.32		
			909.29		
			853.42		
			837.43		

Table 14. Matching sequences of some of the peptides present in fractions 1 – 6.

Mass	Sequence match without misscleavages	Sequence match with 1 misscleavage	Sequence match with 2 misscleavages	Sequence confirmed by MS/MS	Protein *
4276.27			LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLR		β-Lactoglobulin
3361.74		GLDIQKVAGTWYSLAMAASDISLLDAQSAPLR			β-Lactoglobulin
2789.45			ALKALPMHIRLSFNPTQLEEQCHI		β-Lactoglobulin
2775.29		KYLLFCMENSAEPEQSLACQCLVR			β-Lactoglobulin
2707.37	VAGTWYSLAMAASDISLLDAQSAPLR				β-Lactoglobulin
2647.20	YLLFCMENSAEPEQSLACQCLVR				β-Lactoglobulin
2313.25				VYVEELKPTPEGDLEILLQK	β-Lactoglobulin
2091.13			IDALNENKVLVLDTDYKK		β-Lactoglobulin
1963.03		IDALNENKVLVLDTDYK			β-Lactoglobulin
1801.02			TKIPAVFKIDALNENK		β-Lactoglobulin
1770.82		FLDDDLTDDIMCVKK			α-Lactalbumin
1746.88			WENGECAQKKIIAEK		β-Lactoglobulin
1658.78	LSFNPTQLEEQCHI				β-Lactoglobulin
1642.73	FLDDDLTDDIMCVK				α-Lactalbumin
1245.58	TPEVDDEALEK				β-Lactoglobulin
1147.58		LDQWLCEKL			α-Lactalbumin
1065.58				VLVLDTDYK	β-Lactoglobulin
1064.44	WENGECAQK				β-Lactoglobulin
1034.49	LDQWLCEK				α-Lactalbumin
933.54				LIVTQTMK	β-Lactoglobulin
916.47	IDALNENK				β-Lactoglobulin
903.56		TKIPAVFK			β-Lactoglobulin
674.42	IPAVFK				β-Lactoglobulin
673.38	GLDIQK				β-Lactoglobulin
653.30	CEVFR				α-Lactalbumin
650.31	ALCSEK				α-Lactalbumin
573.36	IIAEK				β-Lactoglobulin
549.28	IWCK				α-Lactalbumin

* β-Lactoglobulin and α-Lactalbumin from bovine origin.

3.3.3 Pyrazine formation in Maillard model systems of the peptidic fractions and glucose

The results depicted in **Table 15** show that the model systems containing the different peptidic fractions were able to generate 2-methylpyrazine, 2,5-dimethylpyrazine, 2-ethylpyrazine, 2-ethyl-3-methylpyrazine, 2-ethyl-3,5-dimethylpyrazine and 3-ethyl-2,5-dimethylpyrazine. These results are in line with previous model systems containing tryptic hydrolyzed whey (**Table 8**, **Table 10**). In this set of experiments however, the generated amounts of all the alkypyrazines was quite low, considering that the model systems were incubated at a relative humidity of 32%, which was proven to enhance the generation of pyrazines in previous model systems (**Table 11**). Nevertheless, it is clear that the less efficient generation of pyrazines in all these model systems was due to the low quantities of nitrogen-containing compounds in all the different fractions (**Table 12**).

As shown in **Table 15**, fractions 1 and 2 generated statistically similar levels of pyrazines, except for 2-methylpyrazine. However, it should be noted that fraction 2 contained more than double the amount of total amino acids compared to fraction 1. This observation seems to imply that high molecular weight peptides were less efficient pyrazine precursors. It could be suggested that due to the higher molecular weight of the peptides in fraction 1, their reactivity towards glucose was diminished as a result of more intra- and/or inter peptide interactions.

Considerably more alkylpyrazines were detected in fractions 3 and 4, although their total amino acid content was lower than fraction 2, confirming the trend observed before that higher molecular weight peptides seems less efficient alkylpyrazine precursors. Similarly, although fractions 3 and 4 contained a comparable amount of total amino acids, fraction 4 generated significantly more alkylpyrazines, especially 2,5-dimethylpyrazine. Fractions 5 and 6 however, produced little alkylpyrazines, but it should be noted that their total amino acid content was very low (**Table 12**).

Although the fractionation procedure was not optimal, preliminary observations can be drawn from the results. Firstly, it was observed that all the fractions generated pyrazines, even the fractions of higher molecular weight. There is little information regarding the generation of pyrazines in Maillard research model systems containing polypeptides and proteins, while in contrast, most of the Maillard research on proteins is related to glycation reactions and improvement of functional properties (**Section 1.7.5**). Van Lancker et al. have studied the formation of pyrazines from di- and tripeptides. The authors compared their reactivity with that of free amino acids.[120-121] The results have shown that tripeptides are less reactive than dipeptides. At the same time, in most of the model systems containing dipeptides, these were more reactive than free amino acids. The reasons for which amino acids could generate less pyrazines than dipeptides are not investigated in detail. However, Yaylayan et al.[127,157-158] reported on dicarbonyl elongation reactions, which are mediated by amino acids like

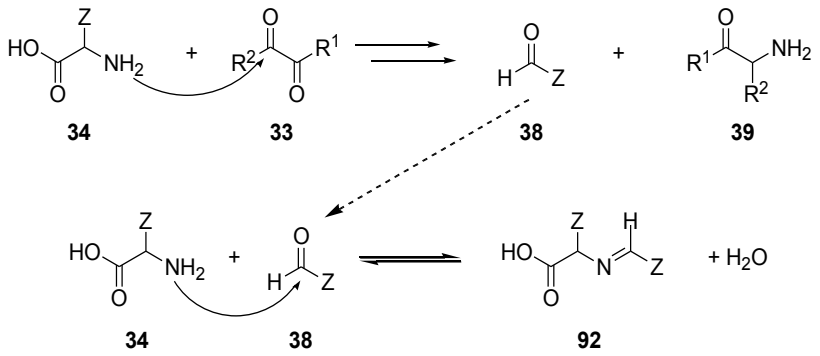
alanine and serine. As amino acids can participate in these reaction generating new dicarbonyl compounds, additional amino acids would be necessary to react with the dicarbonyl compounds and generate aminoketones by the Strecker degradation mechanism (**Section 1.7.1**). Therefore, this could be partially responsible for the hindrance in the pyrazine formation in free amino acid model systems as compared to dipeptides.

Table 15. Pyrazines (GC-MS peak Area x 10^{6}) detected in model reactions of glucose and peptidic fractions 1 - 6 of tryptic hydrolyzed whey protein under roasting conditions (180 ^{0}C/90 min).

Volatile	Frac 1	Frac 2	Frac 3	Frac 4	Frac 5	Frac 6
	Areas x 10^{6}					
Methylpyrazine [A]	2.60 ± 0.18 a	5.99 ± 0.05 b	12.77 ± 0.93 c	15.49 ± 0.47 d	0.88 ± 0.14 e	2.91 ± 0.12 a
2,5-Dimethylpyrazine [A]	4.93 ± 0.67 a	4.25 ± 1.69 a	30.24 ± 0.75 b	80.29 ± 1.72 c	0.77 ± 0.17 d	6.83 ± 0.18 a
Ethylpyrazine [A]	0.62 ± 0.01 a	0.83 ± 0.39 a	2.37 ± 0.21 b	2.48 ± 0.13 b	0.16 ± 0.01 c	0.86 ± 0.02 a
2-Ethyl-3-methylpyrazine [A]	0.56 ± 0.04 a	1.03 ± 0.47 ab	1.92 ± 0.02 c	2.29 ± 0.12 d	0.20 ± 0.01 e	0.71 ± 0.01 a
3-Ethyl-2,5-dimethylpyrazine [A]	0.69 ± 0.08 a	0.93 ± 0.40 a	4.92 ± 0.79 b	10.68 ± 1.06 c	N/D	0.78 ± 0.03 a
2-Ethyl-3,5-dimethylpyrazine [A]	0.68 ± 0.06 a	0.75 ± 0.33 a	2.32 ± 0.14 b	5.65 ± 0.19 c	N/D	0.74 ± 0.03 a

N/D (not detected). [A] Identification confirmed by GC retention index and mass spectra of authentic compounds. Data points represent mean values of 3 independent determinations. Values in the same line followed by different superscript letters are significantly different ($p < 0.05$).

Further, during the Strecker degradation, the generation of the aminoketones **39** is accompanied by the formation of Strecker aldehydes **38**, which are very reactive and can further participate in other reactions. For example, these aldehydes are responsible for specific substitutions in the structure of some pyrazines, like for 2,5-dimethyl-3-(3-methylbutyl)pyrazine. In this case, the 3-methylbutyl-substituent is generated upon an aldol-type reaction of a dihydropyrazine anion with 3-methylbutanal which is the Strecker aldehyde of leucine (**Scheme 6, Section 1.7.1**). Additionally, the previously formed Strecker aldehydes **38** can compete with the dicarbonyl compounds **33** for the amino group of the amino acids. The result of this combination would end in the formation of an unstable imine **92**, which would finally hydrolyze into an amino acid and the same Strecker aldehyde **38**. However, this could possibly slow down the tendency to produce aminoketones **39 (Scheme 14)**.
In the case of peptides, the generation of aminoketones is followed by the formation of a ketoamide complex (**Section 1.7.3**). This ketoamide could also compete with dicarbonyl compounds for the amino group of the *N*-terminal amino acid of the peptide. However, the ketoamide is expected to be less reactive than the dicarbonyl compounds, except if it has a free aldehyde group. Nevertheless, this formation could only happen if the *N*-terminal amino acid of the peptide leading to its formation of the ketoamide is glycine.



Scheme 14 Proposed competition between a Strecker aldehyde and a dicarbonyl for the amino group of an amino acid.

Considering these previously exposed mechanistic differences, it is possible to predict that fractions 5 and 6 would have generated high amounts of pyrazines if their protein content were similar to those of fractions 1 – 4.

3.4 CONCLUSIONS

The results demonstrate that all the collected fractions were able to generate pyrazines, especially the fraction in which peptides were detected using MALDI-TOF-MS analysis in the range of 837 – 180 Da. The formation of pyrazines was hindered as the fractions contained higher molecular weight peptides or had a low content of protein. The results suggest that peptides with a molecular weight above 3000 Da are not very effective precursors, yet the reasons for this are not clear. Peptides with molecular weight below 1000 Da lead to an increased formation of pyrazines.

Additionally, since the peptides present in the fractions have different *N*-terminal amino acids, it is evident that in model systems containing tryptic-hydrolyzed whey protein, the influence of the *N*-terminal amino acid is of a less importance as compared to other factors.

The present chapter investigated the formation of pyrazines in model systems using peptides of different molecular weight that were obtained by GPLC fractionation. The following chapter has focused on studying the generation of pyrazines in model systems containing different peptides generated with different peptidases.

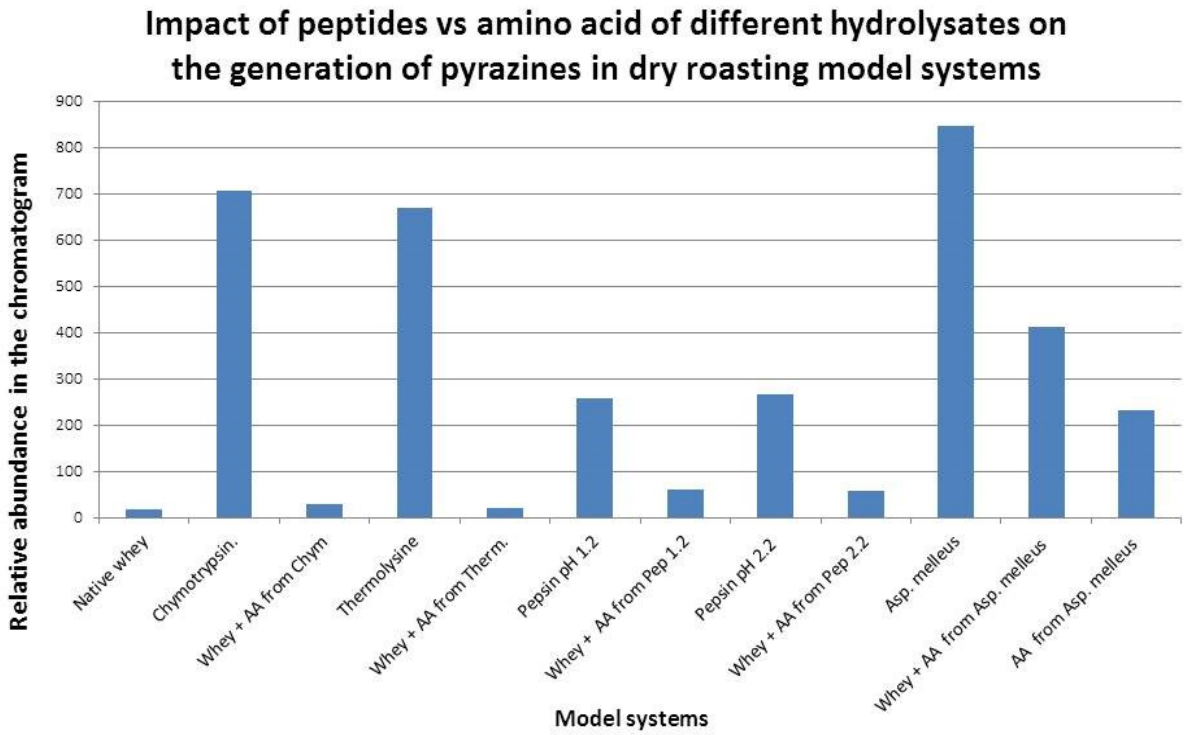
CHAPTER 4

Impact of different enzymatic hydrolysates of whey protein on the formation of pyrazines in Maillard model systems

ABSTRACT

Pyrazines are important volatile Maillard flavor compounds, which contribute to the flavor of many foods producing roasted, earthy, and meat-like aromas. Most studies concerning the generation of pyrazines in Maillard model systems focused on amino acids, di- and tripeptides, while little information is available regarding the impact of oligopeptides. The present study investigated the generation of pyrazines in model systems containing hydrolyzed whey protein under dry heating conditions. Hydrolysates were obtained with different peptidases and were characterized by GPLC and HPLC. The impact of each hydrolysate on the generation of flavor volatiles was measured by HS-SPME-GC/MS. The presence of oligopeptides had an enhancing role in the generation of pyrazines while in contrast, free amino acids contributed to a lesser extent. Additional experiments were considered using α-dicarbonyl compounds in order to elucidate the mechanisms of pyrazine formation. This study demonstrates that oligopeptides are important Maillard flavor precursors.

KEYWORDS: peptides, whey protein hydrolysates, Maillard reaction, pyrazines, HS-SPME-GC/MS, GPLC.



Free amino acids	Chymotrypsin hydrolysate	Thermolysin hydrolysate	Pepsin pH 1.2 hydrolysate	Pepsin pH 2.2 hydrolysate	*Aspergillus melleus* hydrolysate
Mg/100mg					
Total	0.938	0.692	0.715	1.054	23.653

Graphic Abstract 3. Summarized information of **Chapter 4**

4.1 INTRODUCTION

Substituted pyrazines and pyrazine itself are specific Maillard reaction products that contribute significantly to the unique roasted, nutty, meaty, earthy and popcorn-like aroma of many heated food products.[5,7]

Although it has been shown before that peptides may generate pyrazines in the Maillard reaction, their potential contribution to the overall pyrazine formation in food has been investigated to a lesser extent. The generation of pyrazines in Maillard model systems under different conditions and reactants was investigated in **Chapter 2**. It was found that peptides were responsible to a great extent for the generation of pyrazines, while the contribution of free amino acids was rather low. Moreover, in **Chapter 3** it was shown that the impact of peptides depended upon the molecular weight distribution. GPLC fractionation was the method to obtain different peptides for the model systems presented in **Chapter 3**. However, this study aimed to evaluate the impact of different enzymatic whey hydrolysis products on the formation of pyrazines in a Maillard reaction model with glucose.

The chemistry of pyrazine formation in model systems containing proteins, peptides and amino acids needs further study. Therefore additional model systems were made using whey protein hydrolysates and two α-dicarbonyl compounds, methylglyoxal and pentane-2,3-dione, thereby trying to discriminate between the role of free amino acids and peptides generated, respectively, and at the same time elucidating the mechanisms of formation of pyrazines as the source of dicarbonyl compounds is limited. In addition, the impact of the ratio of reagents on the formation of pyrazines was investigated.

4.2 MATERIALS AND METHODS

4.2.1 Chemicals

DL–norvaline (99%), L–cysteine (99.5%), L–4-hydroxyproline (99%), DL–valine (99%), DL–alanine (99%), L–tryptophan (99.5%), L–citruline (99%), sarcosine (99%), DL–histidine (99%), L–isoleucine (99.5%), DL–leucine (99%) and glutamine (99,5%) were purchased from Fluka (Sigma–Aldrich, Bornem, Belgium). DL–lysine monohydrochloride (98%), DL–methionine (99%), glycine (99.5%), L–arginine hydrochloride (99%), L–phenylalanine (99%), L–glutamic acid (99%), D-(+)-glucose (99.5%), trypsin from porcine pancreas, trypsin-chymotrypsin inhibitor from glycine max (soybean)*, insulin from bovine pancreas*, insulin chain B oxidized from bovine pancreas*, cytochrome C from equine heart*, vitamine B_{12}*, Val-Tyr*, proteinase from *Aspergillus melleus,* pepsin from porcine gastric

mucosa, α-chymotrypsin from bovine pancreas, thermolysin from *Geobacillus stearothermophilus*, pyrazine (99%), pyruvaldehyde 40 wt. % solution in water, and 2-methylpyrazine (99%) were purchased from Sigma–Aldrich (Bornem, Belgium). L–lysine (97%), arginine (98%), proline (99%), asparagine monohydrate (99%), 2,5-dimethylpyrazine (99%), 2-ethylpyrazine (98%), 2-ethyl-3-methylpyrazine (99%), 2-ethyl-3,5(6)-dimethylpyrazine (99.5%), 2,3-pentanedione (97%) and trichloroacetic acid (99%) were purchased from Acros Organics, Thermo – Fisher Scientific (Erembodegem, Belgium). Aspartic acid (99%), DL–threonine (99%) and L–tyrosine (99%) were purchased from Merck (Darmstadt, Germany). DL–serine, 2,3-dimethylpyrazine (99%) and 2,6-dimethylpyrazine (96%) were purchased from Janssen Chimica (Geel, Belgium). Magnesium chloride hexahydrate (99%) and sea sand (acid washed and calcinated a.r.) were purchased from Chemlab Analytical (Zedelgem, Belgium). Whey protein isolate LACPRODAN DI-9224 was donated by Arla Foods (Aarhus, Denmark).

* Reagents used as molecular weight markers.

4.2.2 Hydrolysis of whey protein

Whey protein isolate (84% protein) was dissolved in potassium phosphate buffer 0.1M pH 7.8 at a concentration of 5.0 mg/ml, heated at 95 ^{o}C for 5 min and then cooled to at room temperature. The whey protein solution was divided into five aliquots. The pH was adjusted using 2M sodium hydroxide or 2M hydrochloric acid for optimal enzymatic activity. The following pH adjustments were made:

(1) pepsin at pH 1.2, (2) pepsin at pH 2.2, (3) chymotrypsin at pH 8.0, (4) thermolysin at pH 8.0 and (5) proteinase from *Aspergillus melleus* at pH 7.5.

After reaching the desired pH values for optimal enzymatic activity, different enzyme were added at a ratio of 1:20 (w:w) according to the literature.[159]

Samples were incubated at 37 ^{o}C in a water bath during 20h, and the obtained solutions were heated at 95 ^{o}C for 5 min to inactivate the enzymes. The pH of the hydrolysates was adjusted back to 7.8, and the samples were frozen for posterior use. The protein hydrolysates were furthermore characterized with gel permeation chromatography coupled with UV detection at 214 nm. In addition, their free amino acid profile was determined.

4.2.3 Gel permeation chromatography

Samples of whey protein and hydrolyzed whey protein were analyzed as described in **Section 2.2.3**.

4.2.4 Free amino acid analysis

Samples of the different hydrolysates were analyzed to determine their free amino acid profiles. The procedure was followed as described in **Section 2.2.4**. Samples were analyzed in triplicate and the data values depicted in **Table 17** represent the mean value of these analysis

4.2.5 Reactant type and optimal ratio between reagents

For each of the hydrolysates, five different hydrolysate – glucose mixtures were prepared ranging from 1:0.33 to 1:1 (w:w), in order to evaluate the yields of pyrazine formation under roasting conditions (**Section 2.2.5.2.2**). The amount of hydrolyzed whey protein (15 mg) were kept constant while the amount of glucose were added as indicated in **Table 18**. The quantities of hydrolyzed whey and glucose were respectively added from 5.0 mg/ml and 15.0 mg/ml solutions in potassium phosphate buffer 0.1M pH 7.8. All different samples were mixed in a volumetric flask and their final volume was adjusted to 5.0 ml. Then, this mixture (5.0 ml) was transferred to a 20.0 ml SPME vial containing 1.5 g of sand. Subsequently, the samples were frozen and freeze-dried for three days in a VaCo5 freeze-dryer (Zirbus Technology, Bad Grund, Germany) to ensure sample homogeneity (**Section 2.2.5.2.2**).

The freeze-dried samples were subsequently transferred to a hermetic plastic recipient containing a saturated solution of magnesium chloride (32% RH) and incubated during seven days at 30 ^{o}C in an incubator (Memmert, Fisher Scientific, Erembodegem, Belgium) in order to reach a stable a_w in each sample. After reaching equilibrium, the samples were closed with pressure caps and heated in an oven (Memmert, Fisher Scientific) at 180 ^{o}C for 90 min.

4.2.6 Elucidation of the role of different protein hydrolysis products on the formation of pyrazines

Four groups of model systems were prepared as follows for the protein hydrolysates obtained after the enzymatic digestion with pepsin at two different pH values, thermolysin and chymotrypsin:

(1) 15.0 mg of hydrolyzed whey protein and 7.5 mg of glucose, (2) 15.0 mg of native whey protein and 7.5 mg of glucose, (3) 15.0 mg of native whey protein, 7.5 mg of glucose and the addition of a mixture of free amino acids (AA) corresponding to the free amino acid content of the different enzymatic hydrolysates of whey protein as reported in **Table 17**, (4) a mixture of free amino acids corresponding to the free amino acid content of the different enzymatic hydrolysates of whey protein, as reported in **Table 17** and 7.5 mg of glucose.

In the case of the protein hydrolysate obtained with proteinase from *Aspergillus melleus* six model systems were prepared as follows:

(1) 15.0 mg of hydrolyzed whey protein and 7.5 mg of glucose, (2) 15.0 mg of hydrolyzed whey protein and 10.0 mg of glucose, (3) 15.0 mg of native whey protein and 7.5 mg of glucose, (4) 15.0 mg of native whey protein, 7.5 mg of glucose and the addition of a mixture of free amino acids corresponding to the free amino acid content of the enzymatic hydrolysates of whey protein as reported in **Table 17**, (5) 15 mg of native whey protein, 10.0 mg of glucose and the addition of a mixture of free amino acids corresponding to the free amino acid content of the enzymatic hydrolysates of whey protein as reported in **Table 17**, (6) a mixture of free amino acids corresponding to the free amino acid content of the enzymatic hydrolysates of whey protein, as reported in **Table 17**, and 7.5 mg of glucose.

The quantities of native whey protein and hydrolyzed whey protein were respectively added from 5.0 mg/ml solutions in potassium phosphate buffer 0.1M pH 7.8. The quantities of glucose were taken from a 15.0 mg/ml solution in potassium phosphate buffer 0.1M pH 7.8

The quantities of free amino acid were added from different solutions in potassium phosphate buffer 0.1M pH 7.8 which were made following the same methodology which is described in **Table 6** (**Section 2.2.6**)**.** Further, the different reagents were mixed in a volumetric flask and their final volume was adjusted to 5.0 ml. The reaction mixtures were transferred to 20.0 ml SPME vials containing 1.5 g of sand and subsequently frozen and freeze-dried for three days. The freeze-dried samples were transferred to a hermetic plastic recipient containing a saturated solution of magnesium chloride (32% RH) and kept during seven days at 30 ^{o}C inside an incubator (Memmert, Fisher Scientific). After reaching equilibrium, all samples were capped and heated in an oven (Memmert, Fisher Scientific) at 180 ^{o}C for 90 min.

4.2.7 Maillard model systems containing hydrolyzed whey protein and α-dicarbonyl compounds

Six model systems were prepared for the five different protein hydrolysates as follows:

(1) 15 mg of hydrolyzed whey protein and 2 mg of methylglyoxal, (2) 15 mg of native whey protein isolate and 2 mg of methylglyoxal, (3) a mixture of amino acids corresponding to the free amino acid content of the enzymatic digests of whey protein, as reported in **Table 17**, and 2 mg of methylglyoxal, (4) 15 mg of hydrolyzed whey protein and 5 mg of pentane-2,3-dione, (5) 15 mg of native whey protein isolate and 5 mg of pentane-2,3-dione, (6) a mixture of amino acids

corresponding to the free amino acid content of the enzymatic digests of whey protein, as reported in **Table 17**, and 5 mg of pentane-2,3-dione.

The quantities of hydrolyzed whey protein and glucose of the different mixtures were respectively added from 5.0 mg/ml and 15.0 mg/ml solutions in potassium phosphate buffer 0.1M pH 7.8. Further, they were mixed in a volumetric flask and their final volume was adjusted to 5.0 ml. The reaction mixtures were transferred to 20.0 ml SPME vials containing 1.5 g of sand, subsequently frozen and freeze-dried for three days. The freeze-dried samples were transferred to a hermetic plastic recipient containing a saturated solution of magnesium chloride (32% RH) and kept during seven days at 30 ^{o}C inside an incubator (Memmert, Fisher Scientific). After reaching equilibrium, all samples were capped. Further, for the samples containing methylglyoxal, 10.0 µl of a 0.2 g/ml aqueous solution of methylglyoxal were added, obtaining then a final amount of 2 mg of methylglyoxal in the sample. The 0.2 g/ml solution of methylglyoxal was obtained from the corresponding dilution of the 40 % wt aqueous methylglyoxal solution. In the model systems containing pentane-2,3-dione, 10.0 µl of a 0.5 g/ml aqueous solution of pentane-2,3-dione were added to obtain a final amount of 5.0 mg. In both cases, the solutions where added through the septa of the caps using a 10.0 µl syringe. This procedure was followed because of the volatility of the selected α-dicarbonyl compounds. Posteriorly, the samples were heated in an oven (Memmert, Fisher Scientific) at 180 ^{o}C for 90 min.

4.2.8 HS SPME – GC/MS analysis

The volatiles produced under the different experimental conditions were extracted and analyzed as described in **Section 2.2.8**. It should be noted as well that in an additional experiment (data not shown), the addition of 10 µl of an aqueous solution containing 1mg/ml of 2-methylpyrazine to an empty vial and vials containing non-thermally treated samples, produced almost the same peak areas (119.37 x 10^6 and 118.85 x 10^6, respectively). At the same time, the absence of different heating conditions and sample matrixes allowed to exclude the possibility of a matrix effect.

4.2.9 Statistical analysis

All analyses were done using SPSS Statistics version 22 at a significance level of 95 % (p = 0.050). Data points represent the mean value of three independent determinations (see **Section 2.2.9)**.

4.3 RESULTS AND DISCUSSION

The selection of the hydrolysates was done after evaluating several predictive enzymatic hydrolysis of α-lactalbumin and β-lactoglobulin, which constitute between 70 – 90% of the whey protein.[160-161] The predictive hydrolysis was done using chymotrypsin, thermolysin, pepsin at pH 1.2 and 2.2, and proteinase K. According to two specialized softwares provided by the Universal Protein Resource (UniProt) called "Peptide cutter" and "Peptide mass", the use of these enzymes can generate hydrolysates containing peptides of diverse molecular weight and different free amino acid concentrations.[156] At the same time, the cleavage specificity of some enzymes generates peptides with similar *C*-terminus amino acids like in the case of the hydrolysate obtained with chymotrypsin or similar *N*-terminus amino acids like in the case of the hydrolysate obtained with thermolysin (**Appendix 3**). The predictive hydrolysis with proteinase K was not included in the Appendix since the hydrolysis of the whey protein was done using proteinase from *Aspergillus melleus.* However, both enzymes possess a similar cleavage mechanism since they are alkaline peptidases from the family S8.[58]

4.3.1 Characterization of the hydrolysates

A comparison of the chromatograms of the hydrolysates (**Figures 6 – 11**) indicated that the selection of the peptidases resulted in the generation of different peptidic profiles. The results depicted in **Table 15** show in a semiquantitative way the relative abundance of the peptide distribution levels of each hydrolysate, separated on basis of their molecular weight.

Whey protein digested with chymotrypsin showed two major peaks between 1355 and 3496 Da. Peptides between 280 and 1355 Da and below 280 were observed as well, although not in large quantities (**Figure 7**). In contrast, whey protein, digested with thermolysin, exhibited overlapping peaks in the range of peptides between 1355 and 3496 in moderated amounts, and a major production of peptides between 280 and 1355 Da (**Figure 8**).

As shown in **Figure 9**, the hydrolysate obtained with pepsin at pH 1.2 had the highest levels of undigested protein, while the quantity was lower for thermolysin and pepsin at pH 2.2. This can be explained by the fact that even when pepsin shows activity at this pH, its optimal pH is 2.2.[162] At the same time it was observed that the peptic hydrolysates were the ones that produced major quantities of peptides of molecular weight between 1355 and 3496 Da (**Figure 9 – 10**).

Figure 11 shows that the most intense enzymatic hydrolysis was achieved using the proteinase from *Aspergillus melleus*, obtaining high amounts of peptides with a molecular weight below 1355 Da and below 280 Da, which is due to the low cleavage specificity of this enzyme.[163-164]

Though the lowest molecular weight marker (280 Da) elutes at approximately 40 minutes, some peaks with retention times below 50 minutes are present in the different chromatograms (**Figures 7 – 11**). In addition to the absorbance of the peptide bond at 214 nm, it has been reported that the carboxylic acid moiety of amino acids exhibits a weak absorbance between 200 – 215 nm.[165] Furthermore, tyrosine, tryptophan and phenylalanine present UV absorbance between 200 – 300 nm. Therefore, the free amino acids present in the different hydrolysates might be responsible for the peaks detected after 50 minutes in the chromatograms.

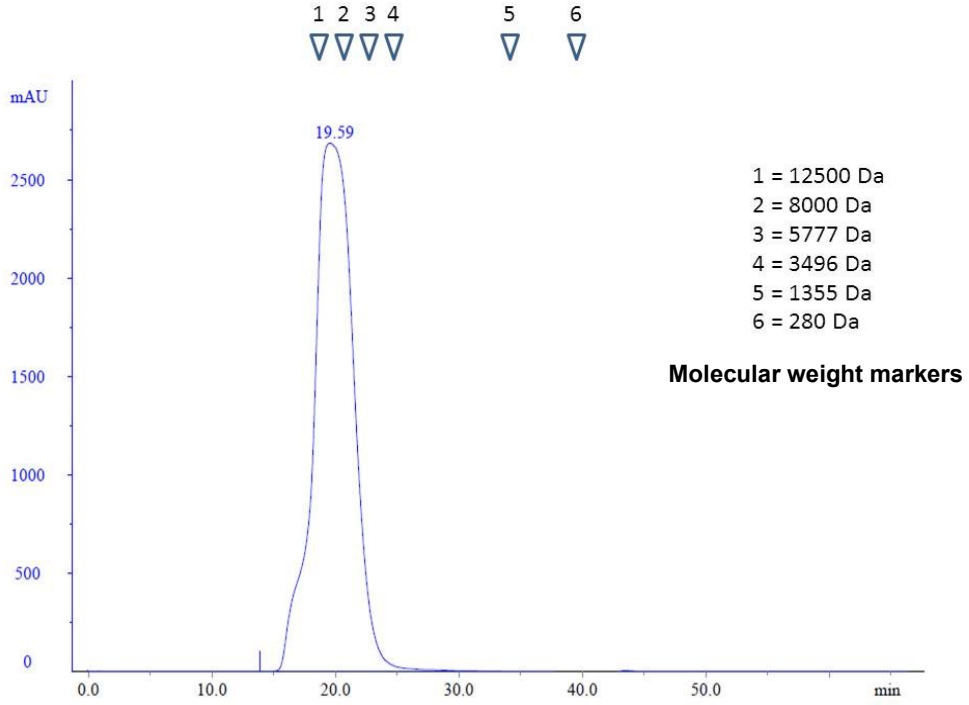


Figure 6. Gel permeation chromatogram of whey protein isolate.

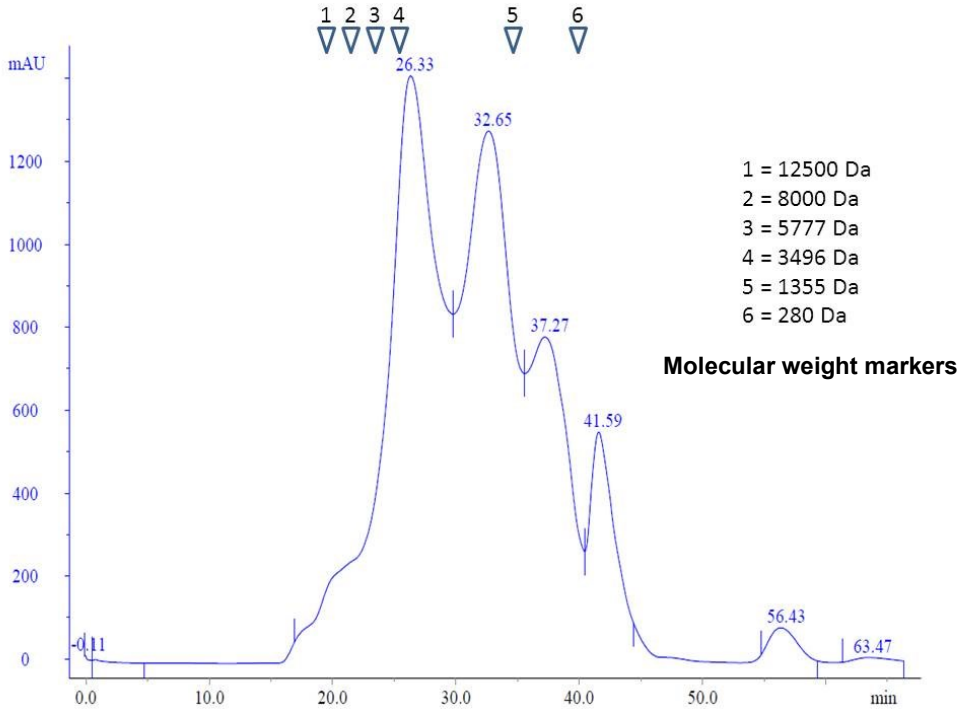


Figure 7. Gel permeation chromatogram of hydrolyzed whey protein after enzymatic digestion with chymotrypsin

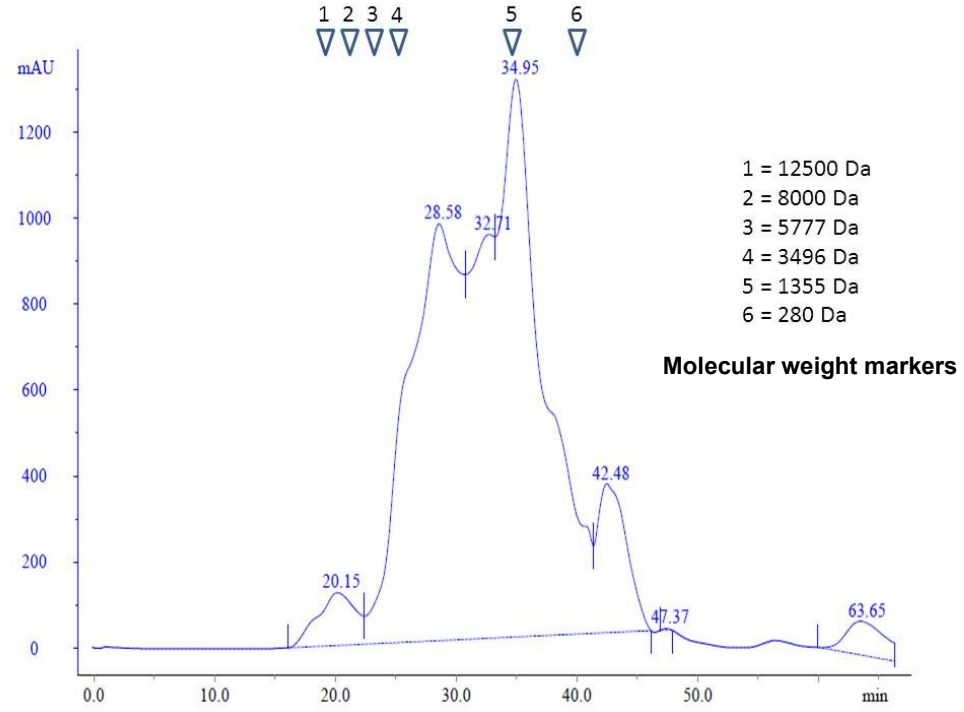


Figure 8. Gel permeation chromatogram of hydrolyzed whey protein after enzymatic digestion with thermolysin

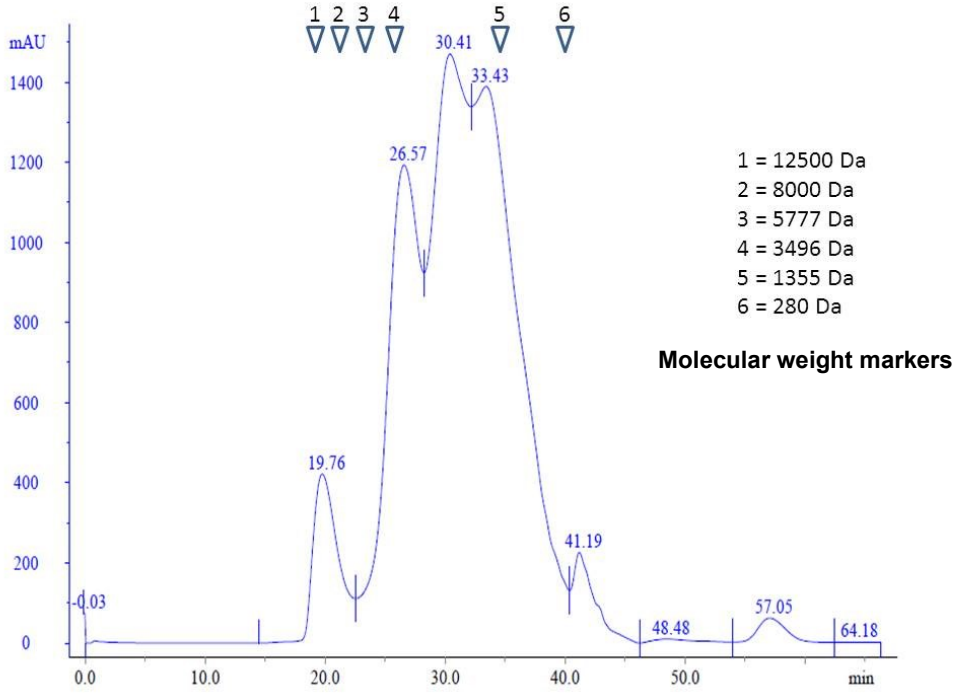


Figure 9. Gel permeation chromatogram of hydrolyzed whey protein after peptic digestion at pH 1.2

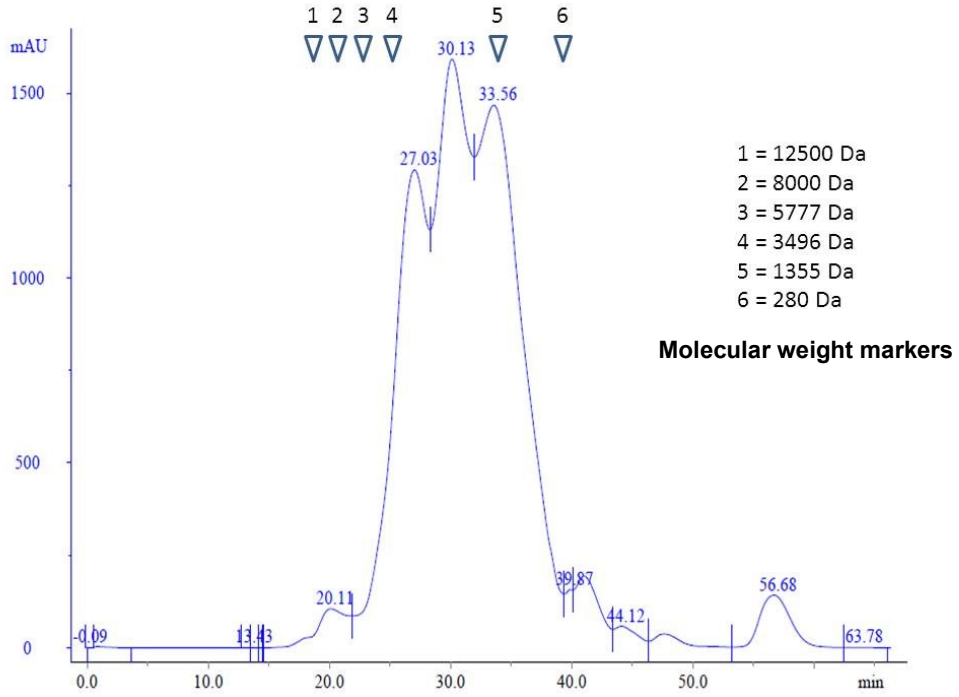


Figure 10. Gel permeation chromatogram of hydrolyzed whey protein after peptic digestion at pH 2.2

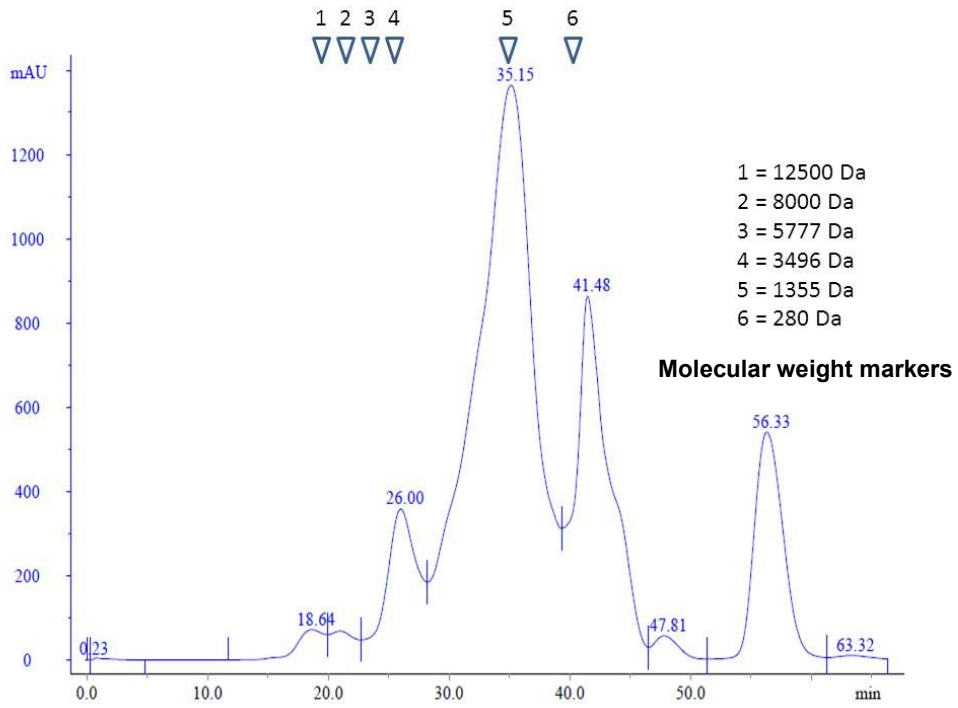


Figure 11. Gel permeation chromatogram of hydrolyzed whey protein after enzymatic digestion with proteinase from *Aspergillus melleus*

Table 16. Peptides detected in different whey protein hydrolysates.

Approximate molecular weight (Da)	Hydrolysate from Chymotrypsin	Hydrolysate from Thermolysin	Hydrolysate from Pepsin pH 1.2	Hydrolysate from Pepsin pH 2.2	Hydrolysate from proteinase of *Aspergillus melleus*
Peak areas expresed in mAU x min (Absorbance at 214 nm)[a]					
> 12500	N/D	478.89 (20.15)	1038.20 (19.76)	311.54 (20.11)	188.42 (18.64)
12500 – 8000	N/D	N/D	N/D	N/D	N/D
8000 – 5777	N/D	N/D	N/D	N/D	167.69 (20.99)
5777 – 3496	N/D	N/D	N/D	N/D	358.663 (26.00)
3496 – 1355	6239.57 (26.33) 4961.32 (32.65)	5056.04 (28.58) 2303.79 (32.71)	3816.21 (26.57) 5087.92 (30.41) 6439.99 (33.43)	4287.35 (27.03) 5115.78 (30.13) 6639.04 (33.56)	N/D
1355 – 280	2331.37 (37.27)	5806.75 (34.95)	N/D	N/D	7735.90 (35.15)
< 280	908.90 (41.59) 170.32 (56.43)	382.20 (42.48) 131.71 (47.37)	488.17 (41.19) 177.45 (57.05)	449.98 (41.02) 123.49 (44.12) 142.27 (56.68)	2907.47 (41.48) 137.03 (47.81) 1506.80 (56.33)

[a] Retention time expressed in minutes is depicted between brackets. N/D: Not detected. Native whey elutes as one unique peak at 16.96 minutes.

The results, presented in **Table 17**, show that the whey protein hydrolysates from chymotrypsin, thermolysin and pepsin at two different pH's produced lower amounts of free amino acids when compared to the hydrolysate obtained with the proteinase from *Aspergillus melleus*. This is obviously due to the fact that the latter proteinase is an exo- and endopeptidase of low specificity which resulted in an enhanced hydrolysis of the whey protein isolate. At the same time, chymotrypsin and thermolysin are known to have a more specific cleavage mechanism.[166] Similar results were obtained previously in whey protein hydrolysates digested with trypsin (**Chapter 2**). Meanwhile, pepsin presents a medium specificity that depends in the pH for its optimal activity pH.[162]

4.3.2 Selection of the reactant ratio of the model systems

In a first series of experiments, the impact of the reactant ratio (hydrolyzed whey protein and glucose) on the formation of pyrazines was evaluated.

Table 17. Free amino acid composition of the different whey protein hydrolysates.

Amino acids	Chymotrypsin	Thermolysin	Pepsin pH 1.2	Pepsin pH 2.2	Proteinase from *Aspergillus melleus*	Trypsin *
Mg/100mg						
Aspartate	N/D	N/D	0.058 ± 0.004	0.036 ± 0.003	0.033 ± 0.003	N/D
Glutamate	N/D	N/D	0.051 ± 0.002	0.046 ± 0.004	0.614 ± 0.033	N/D
Asparagine	0.020 ± 0.001	N/D	N/D	N/D	1.191 ± 0.049	N/D
Serine	0.009 ± 0.001	0.015 ± 0.001	0.012 ± 0.001	0.011 ± 0.001	0.024 ± 0.002	N/D
Glutamine	N/D	N/D	N/D	0.011 ± 0.001	0.730 ± 0.076	N/D
Histidine	N/D	N/D	N/D	N/D	0.679 ± 0.050	N/D
Glycine	0.008 ± 0.001	0.009 ± 0.001	0.007 ± 0.001	0.006 ± 0.001	0.176 ± 0.011	N/D
Threonine	N/D	0.018 ± 0.001	0.012 ± 0.001	0.012 ± 0.001	1.676 ± 0.103	0.039
Arginine	N/D	N/D	N/D	N/D	0.879 ± 0.071	0.055
Alanine	N/D	0.013 ± 0.001	0.019 ± 0.001	0.021 ± 0.001	1.226 ± 0.084	0.010
Tyrosine	0.295 ± 0.004	0.057 ± 0.005	0.071 ± 0.002	0.128 ± 0.005	1.246 ± 0.083	0.042
Valine	N/D	0.017 ± 0.001	0.012 ± 0.001	0.015 ± 0.001	1.875 ± 0.164	0.096
Methionine	0.111 ± 0.001	N/D	0.027 ± 0.001	0.063 ± 0.002	1.103 ± 0.074	0.022
Tryptophane	0.208 ± 0.002	0.094 ± 0.008	0.082 ± 0.002	0.152 ± 0.003	0.701 ± 0.056	0.036
Phenylalanine	0.086 ± 0.004	0.169 ± 0.007	0.133 ± 0.003	0.254 ± 0.009	1.583 ± 0.126	0.030
Isoleucine	N/D	0.057 ± 0.004	0.034 ± 0.001	0.025 ± 0.001	2.442 ± 0.195	0.073
Leucine	0.144 ± 0.001	0.243 ± 0.022	0.197 ± 0.003	0.274 ± 0.009	4.483 ± 0.182	0.134
Lysine	0.057 ± 0.003	N/D	N/D	N/D	2.875 ± 0.104	0.992
Proline	N/D	N/D	N/D	N/D	0.117 ± 0.001	N/D
Total	0.938 ± 0.018	0.692 ± 0.051	0.715 ± 0.023	1.054 ± 0.042	23.653 ± 1.467	1.528

N/D: Not detected (LOD 10 nmols/ml) * Adapted from **Table 5**

The results shown in **Table 18** indicate that during the roasting conditions (180 °C/90 min) significant quantities of the following pyrazines were produced in model systems containing the different whey protein hydrolysates and glucose: 2-methylpyrazine, 2,5(6)-dimethylpyrazine, 2-ethyl-3(5)-methylpyrazine and 3-ethyl-2,5-dimethylpyrazine. At the same time, 2-ethyl-3,5-dimethylpyrazine, 5-ethyl-2,3-dimethypyrazine, 2,5-dimethyl-3-(3-methylbutyl)pyrazine, 2,3-dimethylpyrazine, 2-ethylpyrazine, 2,3-diethyl-5-methylpyrazine and 3,5-diethyl-2-methylpyrazine were produced in smaller quantities. Meanwhile, 2,5-diethylpyrazine and 2-ethyl-3,5,6-trimethylpyrazine were produced in low quantities, and 2-methyl-6-(3-methylbutyl)pyrazine was produced only with the hydrolysates of proteinase from *Aspergillus melleus* and pepsin at both selected pH's. Finally, 2,5-dimethyl-3-(3-methylbutyl)pyrazine, 2,5-dimethyl-3-(2-methylbutyl)pyrazine, 2-isobutyl-3,5,6-trimethylpyrazine were produced only in the model system containing the whey protein hydrolysate from *Aspergillus melleus*. This particular set of pyrazines is known to be produced by specific amino acids (valine, leucine and isoleucine). As observed in **Table 17**, the amount of free amino acids present in the hydrolysate from *A. melleus* proteinase is quite high. Therefore, it is most likely that Strecker aldehydes of the aforementioned amino acids were produced as well. Strecker aldehydes can further react with a dihydropyrazine anion in an aldol-type reaction producing the specific substitution of these pyrazines (**Scheme 5**).

As observed in **Table 18**, 2,5(6)-dimethylpyrazine was produced in high quantities in all model system containing different whey protein hydrolysates, which can be explained by several causes. 2,5-Dimethylpyrazine and 2,6-dimethylpyrazine were considered as one substance, due to the fact that both pyrazines coelute under the selected chromatographic conditions. The mechanism of formation of 2,5-dimethylpyrazine is mainly oxidation of the analogues dimethyl-substituted dihydropyrazine. 2,6-Dimethylpyrazine can be also be formed by this mechanism, however, in the case of 2,6-dimethylpyrazine, the condensation between formaldehyde (Strecker aldehyde from glycine) and the methyl-substituted dihydropyrazine anions is considered as a preferred pathway.[167] Within these pathways, the formation of 2,5- and 2,6-dimethylpyrazine would require C2 and C3 precursors, which are abundant degradation products of carbohydrates.[123,168]

The ratio between hydrolyzed whey protein and glucose played a crucial role in the formation of pyrazines. In general, the generation of pyrazines under dry roasting conditions was relatively low in model systems where glucose was present in small amounts (ratio between hydrolyzed whey and glucose 1:0.33). This effect can be explained by the limiting amount of dicarbonyl compounds generated from glucose.

Higher peak areas for pyrazines were detected for model systems in which the hydrolyzed protein to glucose ratios augmented up to 1:0.5 and 1:0.67. At higher protein to glucose ratios, a decrease in

the formation of pyrazines was observed. This may be due to the excessive formation of different carboxylic acids such as acetic acid from glucose, which might decrease the pH of the model systems.[150] It is generally known that the pH has an impact on the generation of pyrazines,[8] hindering their formation at low pH due to the protonation of the amino groups of the amino acids and peptides.

It was noticed that for the whey protein hydrolysates obtained by enzymatic digestion with chymotrypsin, thermolysin and pepsin at two different pH values, considerably more pyrazines were detected at the protein to glucose ratio of 1:0.5. Similar results were previously reported with tryptic digestions of whey protein isolate (**Chapter 2**).

In the case of the hydrolysate obtained with proteinase from *Aspergillus melleus* the results were less consistent, some pyrazines exhibited higher peak areas in GC/MS chromatograms at a protein to glucose ratio of 1:0.5 and other at 1:0.67. However, previous work presented in **Chapter 2** showed that in model systems containing only lysine and glucose in similar thermal conditions, the optimal lysine to glucose ratio was 1:1.23 and tryptic hydrolyzed whey had an optimal protein to glucose ratio of 1:0.5. Therefore, this shift in the optimal ratio of the protein hydrolysate from *Aspergillus melleus* over glucose for some pyrazines might be due to its high content of free amino acids. In consequence, the hydrolyzed whey protein to glucose ratio of 1:0.5 was further used in subsequent experiments with the hydrolysates of chymotrypsin, thermolysin and pepsin. In addition, two hydrolyzed whey protein to glucose ratios (1:0.5 and 1:0.67) were considered for the hydrolysate from *Aspergillus melleus*.

Table 18. Pyrazines detected in model reactions of hydrolyzed whey protein from enzymatic digestion with chymotrypsin, thermolysin, pepsin at pH 1.2 and 2.2, proteinase from *Aspergillus melleus* (Proteinase As) and glucose under roasting conditions [A]

Pyrazine	Enzymatic hydrolysate	GLU 5mg	GLU 7.5mg	GLU 10mg	GLU 12.5mg	GLU 15mg
2-Methylpyrazine[B]	Chymotrypsin	41.11 ± 0.13 a	40.12 ± 0.77 ab	40.25 ± 0.36 ab	38.93 ± 0.73 b	37.88 ± 0.20 cb
	Thermolysin	27.70 ± 0.97a	44.48 ± 3.73 b	42.05 ± 2.16 b	41.03 ± 1.42 b	40.76 ± 0.16 b
	Pepsin pH 1.2	35.29 ± 1.25 ab	40.91 ± 1.47 c	41.21 ± 1.87 c	38.21 ± 1.56 bc	33.61 ± 0.51 a
	Pepsin pH 2.2	35.29 ± 1.24 a	41.45 ± 1.85 b	41.75 ± 2.35 b	38.21 ± 1.56 ab	33.61 ± 0.51 a
	Proteinase *As*	31.82 ± 2.86 a	35.61 ± 2.24 a	35.13 ± 0.57 a	32.12 ± 1.11 a	25.27 ± 1.70 b
2,5(6)-Dimethylpyrazine[B]	Chymotrypsin	271.37 ± 0.59 a	298.75 ± 5.98 b	261.94 ± 1.33 a	248.91 ± 0.52 c	213.82 ± 1.75 d
	Thermolysin	200.37 ± 13.59 a	279.82 ± 11.34 b	265.51 ± 12.03 b	249.65 ± 17.20 b	246.94 ± 9.14 b
	Pepsin pH 1.2	116.84 ± 1.91 a	117.49 ± 3.52 a	98.03 ± 6.81 b	71.34 ± 3.21 c	57.29 ± 1.57 d
	Pepsin pH 2.2	116.37 ± 1.61 a	112.91 ± 8.25 ab	98.08 ± 6.93 b	71.25 ± 3.10 c	56.71 ± 1.80 d
	Proteinase *As*	213.7 ± 9.13 a	274.86 ± 0.54 b	277.84 ± 13.56 b	271.55 ± 5.21 b	94.86 ± 2.84 c
2,3-Dimethylpyrazine[B]	Chymotrypsin	7.21 ± 0.30 a	7.62 ± 0.03 a	7.81 ± 0.12 ba	6.69 ± 0.07 c	6.63 ± 0.07 c
	Thermolysin	2.97 ± 0.27 a	5.13 ± 0.28 b	5.24 ± 0.14 b	5.38 ± 0.11 b	5.76 ± 0.08 b
	Pepsin pH 1.2	2.82 ± 0.06 a	3.58 ± 0.94 c	3.45 ± 0.25 bc	2.97 ± 0.18 ab	3.08 ± 0.07 abc
	Pepsin pH 2.2	2.70 ± 0.12 ab	3.16 ± 0.10 c	3.04 ± 0.18 bc	2.48 ± 0.58 a	2.60 ± 0.94 a
	Proteinase *As*	3.20 ± 0.18 a	4.68 ± 0.12 b	5.61 ± 0.04 c	5.70 ± 0.04 c	2.62 ± 0.02 d
2-Ethylpyrazine[B]	Chymotrypsin	5.78 ± 0.07 a	5.95 ± 0.02 a	6.73 ± 0.01 b	6.98 ± 0.02 c	7.38 ± 0.10 d
	Thermolysin	5.58 ± 0.37 a	6.83 ± 0.42 b	7.26 ± 0.20 bc	7.48 ± 0.03 bc	8.11 ± 0.43 c
	Pepsin pH 1.2	8.05 ± 0.22 a	10.52 ± 0.07 b	9.85 ± 0.37 bc	9.13 ± 0.11c	9.21 ± 0.18 c
	Pepsin pH 2.2	8.05 ± 0.22 a	10.52 ± 0.07 b	10.32 ± 0.01 b	10.23 ± 0.46 b	10.10 ± 0.26 b
	Proteinase *As*	4.30 ± 0.17 a	5.32 ± 0.35 b	5.89 ± 0.31 b	5.07 ± 0.21 ab	4.49 ± 0.29 a
2-Ethyl-3(5)(6)-methylpyrazine[B]	Chymotrypsin	107.45 ± 1.20 ab	120.78 ± 4.81 c	115.55 ± 2.28 bc	117.10 ± 5.50 bc	102.56 ± 2.04 a
	Thermolysin	73.28 ± 1.85 a	115.26 ± 2.62 b	109.52 ± 1.52 c	104.23 ± 0.71 cd	102.06 ± 0.26 d
	Pepsin pH 1.2	38.81 ± 1.05 ab	42.86 ± 2.86 a	42.43 ± 2.69 a	34.91 ± 1.99 b	33.01 ± 0.73 b
	Pepsin pH 2.2	59.16 ± 1.60 a	62.46 ± 0.94 ab	69.92 ± 1.71 b	84.74 ± 4.66 c	52.76 ± 4.73 a
	Proteinase *As*	102.71 ± 6.44 a	124.48 ± 9.16 ab	129.77 ± 1.53 b	116.79 ± 8.84 ab	68.60 ± 5.91 c
3-Ethyl-2,5-dimethylpyrazine[B]	Chymotrypsin	220.91 ± 18.51 a	215.91 ± 32.51 a	157.36 ± 7.74 c	108.47 ± 36.22 dc	137.72 ± 8.98 dc
	Thermolysin	58.41 ± 2.74 a	87.07 ± 4.04 b	75.26 ± 4.04 c	58.35 ± 0.48 a	56.09 ± 1.23 a
	Pepsin pH 1.2	20.47 ± 0.62 a	15.99 ± 0.71 b	12.52 ± 0.67 c	7.80 ± 0.15 d	6.45 ± 0.12 d
	Pepsin pH 2.2	20.24 ± 0.66 a	15.69 ± 1.08 b	12.09 ± 1.17 c	7.75 ± 0.38 d	6.81 ± 0.37 d
	Proteinase *As*	86.60 ± 2.47 a	117.71 ± 4.92 b	124.89 ± 4.01 b	119.75 ± 1.54 b	18.81 ± 1.19 c
2-Ethyl-3,5-dimethylpyrazine[C]	Chymotrypsin	18.84 ± 0.10 a	23.55 ± 0.44 b	20.04 ± 0.77 c	19.80 ± 0.38 c	15.29 ± 0.15 d
	Thermolysin	7.32 ± 0.38 a	12.97 ± 0.93 b	12.73 ± 0.52 b	11.94 ± 1.02 b	11.94 ± 0.34 b
	Pepsin pH 1.2	4.99 ± 0.06 a	5.44 ± 0.10 a	4.42 ± 0.28 b	3.02 ± 0.06 c	2.46 ± 0.06 d
	Pepsin pH 2.2	5.13 ± 0.12 ab	5.56 ± 0.19 a	4.70 ± 0.25 b	3.29 ± 0.10 c	2.78 ± 0.14 c
	Proteinase *As*	N/D	N/D	N/D	N/D	N/D
Ratio of protein to glucose		1:0.33	1:0.50	1:0.67	1:0.83	1:1

[A] Samples containing hydrolyzed whey protein and glucose were prepared at five different ratios of protein to glucose, ranging from 1:0.33 to 1:1 (w/w),heated at (180^0 C/90 min) and analyzed by HS-SPME-GC/MS. Data points represent mean values of three independent determinations and are given a GC/MS peak area x 10^6. Values in the same row followed by different letters are significantly different ($p<0.05$). [B] Identification confirmed by GC retention index and mass spectra of authentic compounds. [C] Tentatively identified by matching mass spectra library. N/D, not detected

Table 18. Continued.

Pyrazine	Enzymatic hydrolysate	GLU 5mg	GLU 7.5mg	GLU 10mg	GLU 12.5mg	GLU 15mg
5-Ethyl-2,3-dimethylpyrazine[C]	Chymotrypsin	15.71 ± 0.06 a	18.25 ± 0.64 b	14.62 ± 0.47 c	14.28 ± 0.08 c	11.12 ± 0.09 d
	Thermolysin	5.26 ± 0.25 a	10.54 ± 0.32 b	10.56 ± 0.50 b	9.87 ± 0.86 b	9.64 ± 0.26 b
	Pepsin pH 1.2	2.06 ± 0.08 a	2.04 ± 0.04 a	2.01 ± 0.13 ab	1.76 ± 0.05 bc	1.58 ± 0.01c
	Pepsin pH 2.2	2.33 ± 0.12 a	2.48 ± 0.20 a	2.57 ± 0.23 a	2.21 ± 0.13 a	2.24 ± 0.05 a
	Proteinase *As*	26.73 ± 2.46 a	29.83 ± 2.59 a	29.06 ± 2.47 a	23.96 ± 2.12 a	10.02 ± 0.48 b
2,3-Diethyl-5-methylpyrazine[C]	Chymotrypsin	11.76 ± 0.86 a	14.91 ± 0.54 b	10.51 ± 0.59 a	10.19 ± 0.60 a	7.38 ± 0.11 c
	Thermolysin	3.22 ± 0.25 a	9.16 ± 0.40 b	10.40 ± 0.30 c	9.07 ± 0.44 b	7.42 ± 0.36 d
	Pepsin pH 1.2	0.79 ± 0.02 a	0.74 ± 0.03 b	0.71 ± 0.09 b	0.62 ± 0.04 c	0.49 ± 0.01 d
	Pepsin pH 2.2	0.81 ± 0.04 a	0.72 ± 0.04 a	0.70 ± 0.04 ab	0.56 ± 0.06 b	0.56 ± 0.04 b
	Proteinase *As*	12.39 ± 0.80 a	15.47 ± 1.09 b	11.89 ± 0.03 a	9.36 ± 0.52 c	5.05 ± 0.46 d
3,5-Diethyl-2-methylpyrazine[C]	Chymotrypsin	15.63 ± 1.53 a	17.68 ± 0.66 a	10.67 ± 0.53 b	9.51 ± 0.69 bc	6.94 ± 0.20 c
	Thermolysin	3.59 ± 0.24 a	6.18 ± 0.12 b	5.66 ± 0.27 b	4.24 ± 0.02 c	4.20 ± 0.10 c
	Pepsin pH 1.2	1.14 ± 0.02 a	0.78 ± 0.06 b	0.63 ± 0.02 c	0.51 ± 0.01 d	0.43 ± 0.01 e
	Pepsin pH 2.2	1.05 ± 0.03 a	0.82 ± 0.03 b	0.63 ± 0.04 c	0.46 ± 0.03 d	0.44 ± 0.03 d
	Proteinase *As*	9.21 ± 0.37 ab	10.81 ± 0.57 b	8.60 ± 0.02 a	9.87 ± 0.05 b	1.78 ± 0.12 c
2-Ethyl-3,5,6-trimethylpyrazine[C]	Chymotrypsin	7.58 ± 0.29 a	8.20 ± 0.25 b	6.53 ± 0.08 c	6.02 ± 0.08 c	4.37 ± 0.10 d
	Thermolysin	1.76 ± 0.20 a	3.82 ± 0.14 b	3.56 ± 0.12 b	2.69 ± 0.03 c	2.56 ± 0.06 c
	Pepsin pH 1.2	0.65 ± 0.01 a	0.62 ± 0.02 a	0.60 ± 0.08 a	0.47 ± 0.03 b	0.37 ± 0.01 c
	Pepsin pH 2.2	0.62 ± 0.04 a	0.65 ± 0.05 a	0.58 ± 0.02 a	0.45 ± 0.03 b	0.36 ± 0.02 b
	Proteinase *As*	6.21 ± 0.36 a	8.02 ± 0.15 b	7.53 ± 0.34b a	6.87 ± 0.26 a	1.65 ± 0.10 c
2-Methyl-6-(3-methylbutyl)pyrazine[C]	Chymotrypsin	N/D	N/D	N/D	N/D	N/D
	Thermolysin	N/D	N/D	N/D	N/D	N/D
	Pepsin pH 1.2	1.44 ± 0.08 a	1.45 ± 0.01 a	1.48 ± 0.08 a	1.36 ± 0.14 a	1.26 ± 0.05 a
	Pepsin pH 2.2	1.34 ± 0.05 a	1.24 ± 0.53 ab	1.34 ± 0.07 a	1.36 ± 0.02 a	1.11 ± 0.08 b
	Proteinase *As*	15.75 ± 0.31 a	22.25 ± 0.78 b	29.87 ± 2.33 c	25.59 ± 1.78 cb	20.02 ± 0.05 da
2-Isobutyl-3,5,6-trimethylpyrazine[C]	Chymotrypsin	N/D	N/D	N/D	N/D	N/D
	Thermolysin	N/D	N/D	N/D	N/D	N/D
	Pepsin pH 1.2	N/D	N/D	N/D	N/D	N/D
	Pepsin pH 2.2	N/D	N/D	N/D	N/D	N/D
	Proteinase *As*	15.27 ± 0.30 a	15.43 ± 0.30 a	17.48 ± 0.79 b	12.42 ± 0.84 c	5.24 ± 0.28 d
2,5-Dimethyl-3-(2-methyl-butyl)pyrazine[C]	Chymotrypsin	N/D	N/D	N/D	N/D	N/D
	Thermolysin	N/D	N/D	N/D	N/D	N/D
	Pepsin pH 1.2	N/D	N/D	N/D	N/D	N/D
	Pepsin pH 2.2	N/D	N/D	N/D	N/D	N/D
	Proteinase *As*	67.82 ± 0.33 a	71.71 ± 3.30 a	73.06 ± 2.63 a	73.25 ± 3.20 a	25.25 ± 2.47 b
2,5-Dimethyl-3-(3-methylbutyl)pyrazine[C]	Chymotrypsin	31.95 ± 0.87 a	35.73 ± 1.45 b	27.29 ± 0.78 c	23.93 ± 0.69 d	18.38 ± 0.16 e
	Thermolysin	42.14 ± 1.32 a	56.27 ± 3.82 b	52.86 ± 2.46 ba	44.48 ± 3.59 a	37.70 ± 2.72 a
	Pepsin pH 1.2	12.4 ± 0.50 a	8.57 ± 0.28 b	5.52 ± 0.31 c	3.01 ± 0.04 d	2.10 ± 0.04 d
	Pepsin pH 2.2	12.48 ± 0.49 a	8.46 ± 0.48 b	6.13 ± 0.43 c	2.90 ± 0.12 d	2.02 ± 0.09 a
	Proteinase *As*	189.48 ± 3.52 a	198.32 ± 17.59 a	208.26 ± 18.04 a	192.83 ± 14.30 a	141.69 ± 8.97 d
Ratio of protein to glucose		1:0.33	1:0.50	1:0.67	1:0.83	1:1

[A] Samples containing hydrolyzed whey protein and glucose were prepared at five different ratios of protein to glucose, ranging from 1:0.33 to 1:1 (w/w),heated at (180^0 C/90 min) and analyzed by HS-SPME-GC/MS. Data points represent mean values of three independent determinations and are given a GC/MS peak area x 10^6. Values in the same row followed by different letters are significantly different ($p<0.05$). [B] Identification confirmed by GC retention index and mass spectra of authentic compounds. [C] Tentatively identified by matching mass spectra library. N/D, not detected

4.3.3 Elucidation of the role of protein hydrolysis products in the formation of pyrazines

From the previous experiments it was obvious that the different hydrolysates of whey protein were able to generate pyrazines in Maillard reaction model systems. However, the considered hydrolysates are mixtures of different peptides and free amino acids. Therefore, it was not clear whether the compounds responsible for the pyrazine generation were the free amino acids produced during hydrolysis (**Table 17**), or the peptides, or both. Work presented in **Chapter 2** has shown that peptides have a major role in the generation of pyrazines over free amino acids in a mixed system of a tryptic whey hydrolysate and glucose. Nevertheless, the amount and variety of free amino acids present in the tryptic hydrolysate is different from the ones in the current experiment, especially the hydrolysate obtained with proteinase from *Aspergillus melleus*. Therefore, the formation of pyrazines by the whey protein hydrolysates was compared with the pyrazine generation in a mixture of non-hydrolyzed proteins to which the free amino acids, formed upon enzymatic hydrolysis, were added according to the amounts reported in **Table 17**. Non-hydrolyzed whey protein without the addition of free amino acids was included as a control. In addition, glucose together with the free amino acids present in the various protein hydrolysates were considered as well.

As the native whey protein isolate did not contain any free amino acids, the limited generation of pyrazines was probably due to the thermal degradation of the protein under the selected reaction conditions, releasing amino compounds that can react with glucose and form pyrazines. Another possible reason may be due to the formation of ammonia from amino acid residues of native whey protein. Asparagine and arginine can produce ammonia as a consequence of several deamidation reactions, even if these amino acids are incorporated in proteins.[169] The reaction between ammonia and glucose leading to the production of pyrazines has been reported repeatedly (see **Section 1.7**).[75,115,122]

For chymotrypsin, thermolysin and pepsin hydrolysates, the same general trends were observed. The model systems containing their respective free amino acid mixtures and glucose did not generate pyrazines in detectable amounts. It is most likely that the amino acid levels present in these mixtures were not high enough to generate pyrazines. Therefore, these data are not included in **Table 19**. Secondly, model systems containing hydrolyzed whey protein and glucose led to the formation of the highest amounts of pyrazines (**Table 19**), what is in line to the results presented in **Chapter 2.** Therefore, this set of experiments made in Maillard model systems using different whey protein hydrolysates confirms that peptides are important pyrazine precursors.

In the hydrolysate obtained with thermolysin, the generated amount of some pyrazines was slightly lower than in the case of the hydrolysate obtained with chymotrypsin. This difference can be related to the fact that in the hydrolysate obtained with thermolysin, most of the *N*-terminal amino acids are leucine, isoleucine, phenylalanine and valine (**Appendix 3**). It has been reported that the side chain of these amino acids could have a small inhibiting effect in the case of dipeptides due to steric hindrance, thus making the overall reaction slower.[121]

In the case of both peptic hydrolysates, it was expected that they would generate pyrazines in similar levels as the hydrolysates of chymotrypsin and thermolysin, since they have comparable quantities of free amino acids and similar peptidic profiles. Nevertheless, it is likely that the production of pyrazines was inhibited due to the presence of NaCl generated after adjusting the pH of the hydrolysates from 1.2 or 2.2 to 7.8 after the digestion using NaOH, as they were previously acidified with HCl (**Section 4.2.2**). The effect of NaCl on the formation of pyrazines was not reported to the best of our knowledge. However, Gökmen et al.[170] described that Na cations promote the dehydration of glucose generating hydroxymethylfurfural and furfural, and at the same time it inhibits the formation of an imine in the initial stage of the Maillard reaction. Therefore, the formation of the intermediate products that lead to the formation of pyrazines could be hindered.

It was remarkable that 3-ethyl-2,5-dimethylpyrazine was found in higher quantities in the model systems containing glucose and hydrolyzed whey protein obtained after enzymatic digestion with chymotrypsin as compared to the other hydrolysates. However, this hydrolysate and the hydrolysate from *Aspergillus melleus* are the only hydrolysates that contains free lysine. The results presented in **Chapter 2** also exhibit a considerable abundance of 3-ethyl-2,5-dimethylpyrazine in similar experimental conditions (**Table 10**), using a tryptic whey hydrolysate, that also contains free lysine (**Table 5**). Lysine stimulates the formation of pyrazines due to the presence of an additional amino group, and at the same time, it can act as a synergist with respect to the reactivity of other amino acids, although it is not clear how the catalytic mechanism works.[4,113] Therefore, it is clear that the presence of lysine cannot be the only reason for the formation of this pyrazine. In fact, several mechanisms were reported for the formation of this particular pyrazine **(Scheme 15)**.

Table 19. Pyrazines detected in various model reactions of whey protein, hydrolyzed whey protein and glucose

Native whey 15mg	Chym. Hydrolysate 15mg	Whey 15mg + AA from Chymotrypsin digestion	Therm. Hydrolysate 15mg	Whey 15mg + AA from Thermolysin digestion	Pep.1.2 Hydrolysate 15mg	Whey 15mg + AA from Pep 1.2 digestion	Pep.pH 2.2 Hydrolysate 15mg	Whey 15mg + AA from Pep 2.2 digestion	Hydrolyzed Whey from Prot. *Asp. melleus* 15mg	Whey 15mg + AA from Prot. *Asp. melleus* digestion	Hydrolyzed Whey from Prot. *Asp. melleus* 15mg	Whey 15mg + AA from Prot. *Asp. melleus* digestion	AA from Prot. *Asp. melleus* digestion
Glu 7.5mg	Glu 7.5mg	Glu 7.5mg	Glu 7.5mg	Glu 7.5mg	Glu 7.5mg	Glu 7.5mg	Glu 7.5mg	Glu 7.5mg	Glu 7.5mg	Glu 7.5mg	Glu 10mg	Glu 10mg	Glu 7.5mg
2-Methylpyrazine[B]													
3.46 ± 0.11 a	50.68 ± 0.40 f	5.68 ± 0.20 ab	52.27 ± 1.14 f	3.99 ± 0.25 a	40.92 ± 1.47 e	10.03 ± 0.08 b	41.45 ± 1.85 e	10.26 ± 0.46 b	34.75 ± 1.81 d	43.05 ± 3.27 e	35.47 ± 0.80 d	22.99 ± 0.32 c	27.26 ± 1.12 c
2,5 (6)-Dimethylpyrazine[B]													
7.50 ± 0.18 a	248.51 ± 8.76 ef	11.3 ± 0.17 a	297.11 ± 11.32 g	7.96 ± 0.26 a	117.50 ± 3.52 c	25.95 ± 1.44 b	112.91 ± 8.25 c	19.21 ± 1.24 a	231.50 ± 10.44 e	141.01 ± 4.71 d	259.02 ± 4.92 f	132.65 ± 9.39 cd	74.78 ± 5.47 b
2,3-Dimethylpyrazine[B]													
N/D	7.78 ± 0.60 d	N/D	6.04 ± 0.26 e	N/D	3.17 ± 0.10 a	N/D	3.58 ± 0.94 a	N/D	5.83 ± 0.30 b	3.55 ± 0.04 a	5.45 ± 0.53 c	4.45 ± 0.14 a	3.40 ± 0.01 a
2-Ethylpyrazine[B]													
N/D	7.93 ± 0.23 d	N/D	7.98 ± 0.23 d	N/D	10.53 ± 0.07 e	N/D	10.52 ± 0.08 e	N/D	6.46 ± 0.08 c	3.84 ± 0.20 ba	6.86 ± 0.02 c	4.27 ± 0.08 a	4.42 ± 0.26 a
2-Ethyl-3(5)(6)-methylpyrazine[B]													
5.48 ± 0.18 a	120.75 ± 4.80 c	7.18 ± 0.41 a	115.26 ± 2.62 dc	4.92 ± 0.44 a	42.86 ± 2.85 g	16.53 ± 0.74 ea	62.45 ± 0.94 h	20.32 ± 0.06 b	124.48 ± 9.16 c	34.20 ± 2.26 f	129.77 ± 1.53 c	24.45 ± 0.62 b	24.49 ± 0.99 b
3-Ethyl-2,5-dimethylpyrazine[B]													
1.37 ± 0.04 a	167.65 ± 22.34 g	2.27 ± 0.18 a	79.79 ± 3.85 e	1.42 ± 0.08 a	16.00 ± 0.71 c	4.87 ± 0.09 ab	15.69 ± 1.08 bc	3.70 ± 0.20 a	87.56 ± 4.31 ef	21.23 ± 1.28 cd	97.97 ± 1.88 f	28.03 ± 0.93 d	8.43 ± 0.38 ab
2-Ethyl-3,5-dimethylpyrazine[C]													
0.53 ± 0.08 a	16.36 ± 2.07 e	1.17 ± 0.03 ab	13.23 ± 0.86 d	0.66 ± 0.02 ab	5.45 ± 0.10 c	1.96 ± 0.01 ab	5.56 ± 0.19 c	1.62 ± 0.08 ab	N/D	N/D	N/D	N/D	2.84 ± 0.15 b

[A] Data points represent mean values of three independent determinations and are given as GC/MS peak area x 10^6. Values in the same row followed by different letters are significantly different ($p<0.05$). Model reaction were as follows: dry mixtures of different whey hydrolysates + glucose, whey + glucose, free amino acids obtained from different whey hydrolysates as described in **Table 17** + glucose, whey with the addition of an amount of free amino acids obtained from different whey hydrolysates as described in **Table 17** + glucose. [B] Identification confirmed by GC retention index and mass spectra of authentic compounds. [C] Tentatively identified by matching mass spectra library. N/D, not detected

Table 19 continued.

Native whey 15mg	Chym. Hydrolysate 15mg	Whey 15mg + AA from Chymotrypsin digestion	Therm. Hydrolysate 15mg	Whey 15mg + AA from Thermolysin digestion	Pep.1.2 Hydrolysate 15mg	Whey 15mg + AA from Pep 1.2 digestion	Pep.pH 2.2 Hydrolysate 15mg	Whey 15mg + AA from Pep 2.2 digestion	Hydrolyzed Whey from Prot. *Asp. melleus* 15mg	Whey 15mg + AA from Prot. *Asp. melleus* digestion	Hydrolyzed Whey from Prot. *Asp. melleus* 15mg	Whey 15mg + AA from Prot. *Asp. melleus* digestion	AA from Prot. *Asp. melleus* digestion
Glu 7.5mg	Glu 7.5mg	Glu 7.5mg	Glu 7.5mg	Glu 7.5mg	Glu 7.5mg	Glu 7.5mg	Glu 7.5mg	Glu 7.5mg	Glu 7.5mg	Glu 7.5mg	Glu 10mg	Glu 10mg	Glu 7.5mg
5-Ethyl-2,3-dimethylpyrazine[C]													
0.69 ± 0.04 a	11.66 ± 0.63 d	1.24 ± 0.04 a	10.71 ± 0.70 d	0.70 ± 0.04 a	2.05 ± 0.04 ab	1.82 ± 0.01 ab	2.48 ± 0.20 ab	1.64 ± 0.09 a	26.26 ± 2.01 f	5.62 ± 0.63 cb	18.76 ± 0.61 e	3.94 ± 0.22 b	2.60 ± 0.15 b
2,3-Diethyl-5-methylpyrazine[C]													
N/D	14.6 ± 0.48 d	N/D	9.89 ± 0.92 e	N/D	7.42 ± 0.03 ab	N/D	0.72 ± 0.04 a	N/D	8.23 ± 0.71 b	N/D	25.78 ± 0.26 c	1.17 ± 0.22 a	N/D
3,5-Diethyl-2-methylpyrazine[C]													
N/D	16.86 ± 0.93 d	N/D	5.80 ± 0.39 b	N/D	0.79 ± 0.06 a	N/D	0.82 ± 0.03 a	N/D	7.36 ± 0.55 c	N/D	6.57 ± 0.24 bc	1.14 ± 0.19 a	N/D
2-Ethyl-3,5,6-trimethylpyrazine[C]													
N/D	8.27 ± 0.72 e	N/D	3.30 ± 0.25 b	N/D	0.62 ± 0.02 a	N/D	0.65 ± 0.05 a	N/D	6.99 ± 0.40 d	N/D	5.68 ± 0.23 c	N/D	N/D
2-Methyl-6-(3-methylbutyl)pyrazine[C]													
N/D	2.89 ± 0.16 a	N/D	8.52 ± 0.72 b	N/D	1.46 ± 0.01 a	N/D	1.24 ± 0.05 a	N/D	25.34 ± 0.20 e	33.07 ± 0.28 d	64.11 ± 5.11 f	37.98 ± 1.06 d	13.85 ± 0.13 c
2-Isobutyl-3,5,6-trimethylpyrazine[C]													
N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	13.42 ± 0.91 c	1.71 ± 0.02 a	13.45 ± 0.18 c	5.27 ± 0.47 b	N/D
2,5-Dimethyl-3-(2-methylbutyl)pyrazine[C]													
N/D	N/D	N/D	4.66 ± 0.18 a	N/D	N/D	N/D	N/D	N/D	70.93 ± 1.72 d	18.08 ± 0.15 b	74.62 ± 2.88 d	47.70 ± 4.40 c	13.20 ± 0.97 b
2,5-Dimethyl-3-(3-methylbutyl)pyrazine[C]													
N/D	33.71 ± 1.39 g	N/D	54.11 ± 1.65 b	N/D	8.57 ± 0.28 a	N/D	8.46 ± 0.48 a	N/D	197.11 ± 6.73 e	107.93 ± 5.01 c	253.48 ± 4.72 f	229.63 ± 8.37 d	55.96 ± 1.40 b

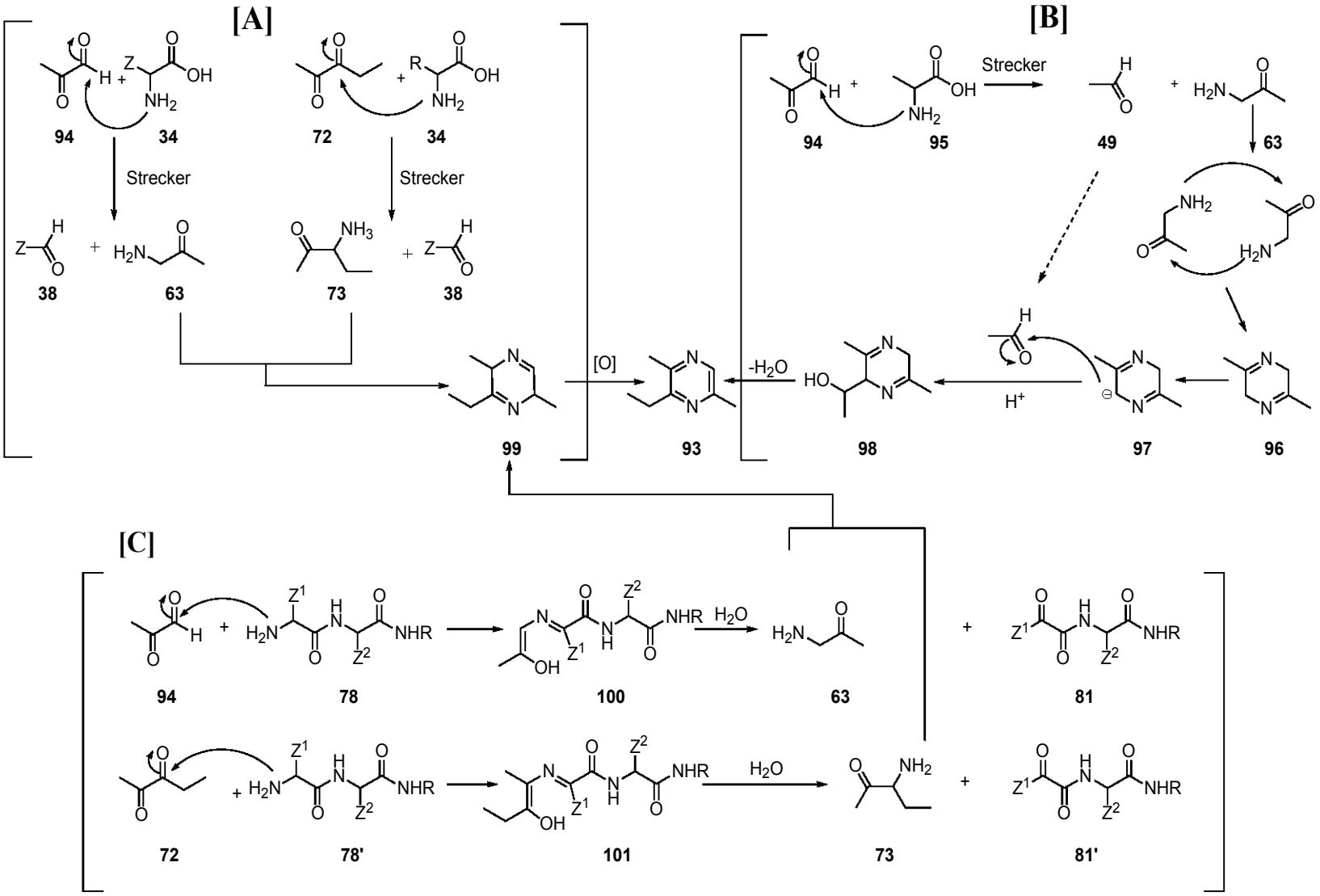
[A] Data points represent mean values of three independent determinations and are given as GC/MS peak area x 10^6. Values in the same row followed by different letters are significantly different ($p<0.05$). Model reaction were as follows: dry mixtures of different whey hydrolysates + glucose, whey + glucose, free amino acids obtained from different whey hydrolysates as described in **Table 17** + glucose, whey with the addition of an amount of free amino acids obtained from different whey hydrolysates as described in **Table 17** + glucose. [B] Identification confirmed by GC retention index and mass spectra of authentic compounds. [C] Tentatively identified by matching mass spectra library. N/D, not detected

3-Ethyl-2,5-dimethylpyrazine **93** could be formed from the condensation of 1-aminopropan-2-one **63** and 3-aminopentan-2-one **73**. Both α-amino ketones **63** and **73** are produced from methylglyoxal **94** and pentane-2,3-dione **72,** respectively, through the Strecker degradation pathway **(Scheme 15 A).** Moreover, pentane-2,3-dione **72** can also generate another α-amino ketone (2-aminopentan-3-one) due to lack of selectivity of the nucleophilic attack on both carbonyl groups. This would result in the formation of 2-ethyl-3,5-dimethylpyrazine. Another possibility is that 3-ethyl-2,5-dimethyl-pyrazine **93** is formed through the reaction between methylglyoxal **94** and free alanine **95** through the Strecker degradation pathway **(Scheme 15 B)**. The resulting 1-aminopropan-2-one **63** can condense to form the 2,5-dimethyldihydropyrazine anion **97** which can further react with acetaldehyde **49** leading to the formation of 3-ethyl-2,5-dimethylpyrazine **93**. Acetaldehyde **49** is produced from either the Strecker degradation of alanine **95** or thermally induced carbohydrate degradation. Third, 1-aminopropan-2-one **63** and 3-aminopentan-2-one **73** can also be formed from peptides **78** in reaction with methylglyoxal **94** and pentane-2,3-dione **72**, respectively, through the α-amino ketone formation pathway[120] **(Scheme 15 C)**.

Since the whey protein hydrolysate obtained with chymotrypsin does not contain alanine **95** (**Table 14**), the formation of 3-ethyl-2,5-dimethylpyrazine **93** is not likely to be formed through the second mechanism stated above (**Scheme 15 B**). Hence, 3-ethyl-2,5-dimethylpyrazine is more likely to be generated by a combination of the first and third formation mechanism (**Scheme 15 A and C**). Additionally, 3-ethyl-2,5-dimethylpyrazine **93** can be formed via thermally induced autocondensation of 1-aminopropan-2-one **63**, as reported by Rizzi et al.[124]

The *Aspergillus melleus* hydrolysate produced high levels of 3-ethyl-2,5-dimethylpyrazine as well. It is obvious that this hydrolysate contained a significant amount of alanine **95**, which can produce acetaldehyde **47** through the Strecker degradation pathway. As mentioned before, acetaldehyde **47** can condense with the 2,5-dimethyldihydropyrazine anion **97** leading to the formation of 3-ethyl-2,5-dimethylpyrazine **97**. For the rest of the hydrolysates, the amount of 3-ethyl-2,5-dimethylpyrazine was found to be lower, especially for pepsin.

The hydrolysate from Asp. *melleus* generated more pyrazines that the model containing native whey with the addition of the free amino acids generated upon Asp. *melleus* proteinase hydrolysis. However, compared to the other enzymes, the free amino acid mixture generated from the Asp. *melleus* proteinase hydrolysis was able to generate substantial levels of pyrazines by itself if reacted in the presence of glucose.



Scheme 15. Mechanisms of the formation of 3-ethyl-2,5-dimethylpyrazine: [A] & [B]: amino acid + dicarbonyl compounds, [C]: peptides + dicarbonyl compounds. Adapted from Van Lancker et al.[121] [O]: oxidation

This illustrates that for this hydrolysate in particular, free amino acids had an important role since they are present in high amounts when compared to the other hydrolysates. Similarly, the high amounts of amino acid specific pyrazines present in these samples is a direct consequence of the high concentration of free amino acids, especially leucine. In contrast, the rest of the hydrolysates produced small amount of amino acid specific pyrazines. This is due to the fact that peptides cannot follow the Strecker degradation, therefore the formation of the α-amino ketone is followed by the formation of a complex α-ketoamide instead of a Strecker aldehyde.[9]

An exception to consider was the hydrolysate obtained from the digestion with thermolysin in which moderate amounts of 2-methyl-6-(3-methylbutyl)pyrazine were detected among with considerable quantities of 2,5-dimethyl-3-(3-methylbutyl)pyrazine. This result can be due to the fact that this hydrolysate contained slightly higher quantities of leucine and isoleucine than the rest of the hydrolysates with the exception of those obtained with proteinase from *Aspergillus melleus* (**Table 14**).

4.3.4 Maillard model systems containing hydrolyzed whey protein and two different dicarbonyl compounds

Novotny et al.[123] studied the formation of α-dicarbonyl compounds during the degradation of monosaccharides and found significant quantities of glyoxal and methylglyoxal. Meanwhile, ethylglyoxal, butane-2,3-dione and pentane-2,3-dione were found as minor products of the degradation of glucose. These α-dicarbonyl compounds are important precursors in the formation of α-amino ketones when they react with amino acids or peptides. The posterior condensation of these α-amino ketones can yield several substituted pyrazines. However, it is also known that when α-dicarbonyl compounds react with free amino acids to form α-amino ketones through the Strecker degradation, Strecker aldehydes are produced. These Strecker aldehydes can further participate in the formation of pyrazines by reacting with a dihydropyrazine anion, in an aldol type reaction before its final oxidation step generating then a specific alkyl substituent in the pyrazine.[120] For example, the mechanism of formation of 3-ethyl-2,5-dimethylpyrazine, which was described earlier (**Scheme 15**), illustrates clearly that one particular pyrazine can be generated upon different pathways. Since hydrolyzed whey protein contains both free amino acids and peptides, it was necessary to design an experiment to elucidate the formation mechanism of pyrazines, more specifically if the substituents on the pyrazine are originated from free amino acids, peptides, or the combined sources. An optimal experimental design would be by using isotope labeled peptides and amino acids. Nevertheless, the complex composition of peptides from the different hydrolysates made this approach difficult to be

applied. Therefore, model systems containing whey hydrolysates and model systems containing mixtures of free amino acids, were prepared by restricting the present α-diones to one, thus limiting the variety of pyrazine formation.

Methylglyoxal and pentane-2,3-dione were selected to be used in the model systems. Methylglyoxal was suitable to be used because it is produced in high amounts as a degradation product of monosaccharides.[123] Therefore, it can be related to the previous used model systems containing whey protein hydrolysates and glucose. At the same time, the use of methylglyoxal for Maillard model systems would generate 1-aminopropan-2-one as the only possible α-aminoketone. Hence, this would allow to identify the origin of additional substituents on the pyrazines. Pentane-2,3-dione was selected as less reactive α-dicarbonyl compound which is also produced as a degradation product of glucose. It would produce two different α-aminoketones (3-aminopentan-2-one and 2-aminopentan-3-one), whose condensation would result in methyl and ethyl substituents in position 2 and 3 of the pyrazines. This limited formation of substituted pyrazines would allow identifying additional formation mechanisms of pyrazines. Hence, Maillard model systems were prepared for all the previously obtained hydrolysates, adding in one case methylglyoxal as α-dicarbonyl source and pentane-2,3-dione in the second.

The quantities of α-diones were selected after comparing which was the lowest concentration that would form pyrazines in similar amounts as in model systems containing protein hydrolysates and glucose. However, since these quantities were weight-based they are not equimolar.

The results (**Table 20**) showed that native whey generated the lowest quantities of pyrazines in its corresponding model system with both dicarbonyl compounds. It should be noted that pentane-2,3-dione and methylglyoxal were not detected with these mixtures, suggesting that they could have reacted with side amino groups of the protein. In addition, both dicarbonyls are known to be very reactive, leaving the possibility of selfcondensation reactions.

At the same time it was observed that in model systems containing only free amino acids corresponding to the values of **Table 17** for chymotrypsin, thermolysin and pepsin hydrolysates, there was still unreacted pentane-2,3-dione. However, it was not detected in the other model systems, showing that pentane-2,3-dione did not degrade under the selected thermal conditions. As no methylglyoxal was detected in all different model systems it was concluded that it was consumed during the reactions.

For all different hydrolysates and amino acid mixtures, methylglyoxal generated higher amounts of pyrazines than pentane-2,3-dione, except for 2,5-diethyl-3,6-dimethylpyrazine and 2,6-diethyl-3,5-dimethylpyrazine. These pyrazines could be formed by the reaction of pentane-2,3-dione with amino acids or peptides resulting in the formation of their respective α-amino ketones.

2,5(6)-Dimethylpyrazine was found to be the most abundant pyrazine for all model systems, especially those in which methylglyoxal was used. It was particularly interesting to find 2,5(6)-dimethylpyrazine in model systems with pentane-2,3-dione since the dione was found to be not fully consumed during the reaction, and at the same time, it is not a precursor for the formation of this pyrazine. However, threonine and serine can form pyrazines during roasting conditions without the participation of any dicarbonyl compounds due to formation of α-amino ketones via decarbonylation, decarboxylation and dehydration of both amino acids (**Scheme 8-9**).[126]
Since threonine is a quite abundant amino acid present in the hydrolysate of whey protein obtained with proteinase with *Aspergillus melleus*, it is obvious that this proposed mechanism was at least partially responsible for the high quantities of 2,5(6)-dimethylpyrazine found in this particular hydrolysate or its corresponding amino acid mixture. At the same time, 2,5(6)-dimethylpyrazine was not detected in the other free amino acid-containing model systems. This is probably because threonine was present in negligible amounts.

With pentane-2,3-dione, relative small amounts of 2,5(6)-dimethylpyrazine were detected in model systems of native whey protein and whey protein hydrolysates, suggesting a thermally induced degradation of the peptides and whey protein allowing the formation of precursors for this pyrazine.
It is still quite remarkable that both model systems containing only the mixture of amino acids, generated upon the hydrolysis of whey protein with proteinase from *Aspergillus melleus*, yielded less 2,5(6)-dimethylpyrazine than the rest of the amino acid mixtures corresponding to the other hydrolysates. Meanwhile, this pyrazine was more abundant in the *Aspergillus melleus* hydrolysate than in the other hydrolysates. However, methylglyoxal or pentane-2,3-dione were neither detected in the model system of the whey protein hydrolysate of proteinase from *Aspergillus melleus*, nor in the corresponding amino acid mixture. Nevertheless, those are the systems that generated the highest amounts of amino acid specific pyrazines [2,5-dimethyl-3-(3-methylbutyl)pyrazine and 2,5-dimethyl-3-(2-methylbutyl)pyrazine]. Both are generated with the participation of the 2,5-dihydropyrazine (anion), which reacts in an aldol type reaction with Strecker aldehydes to form these amino acid specific pyrazines.

Table 20. Pyrazines detected in various model reactions of hydrolyzed whey protein, amino acids and α-dicarbonyls[A]

M.glyoxal 2mg Pent.-2,3-dione 5mg	Native Whey 15mg	Chym. Hydroly-sate 15mg	AA from Chym. digestion	Therm. hydroly-sate 15mg	AA from Therm. digestion	Pep.1.2 hydroly-sate 15mg	AA from Pep 1.2 digestion	Pep.2.2 hydroly-sate 15mg	AA from Pep 2.2 digestion	*A. melleus* hydroly-sate 15mg	AA from Prot. *Asp. melleus* digestion
2,5 (6)-Dimethylpyrazine[B]											
M.glyoxal	15.14 ± 7.09 a	43.11 ± 12.33 b	181.23 ± 9.98 d	36.37 ± 5.09 ba	174.23 ± 5.76 d	43.66 ± 5.75 b	231.87 ± 10.89 e	53.43 ± 13.71 b	183.09 ± 28.86 d	90.20 ± 9.08 cb	59.81 ± 12.88 b
Pent.-2,3-dione	6.46 ± 0.31 a	N/D	N/D	10.30 ± 0.26 ba	N/D	10.30 ± 0.21 ba	N/D	15.65 ± 1.01 ba	N/D	46.76 ± 19.96 c	67.74 ± 2.49 c
2-Ethyl-3(5)(6)-methylpyrazine[B]											
M.glyoxal	N/D	N/D	N/D	4.81 ± 0.13 a	N/D	4.42 ± 0.16 a	N/D	7.15 ± 0.30 b	N/D	N/D	N/D
Pent.-2,3-dione	N/D	N/D	N/D	4.16 ± 0.28 a	N/D	N/D	N/D	5.60 ± 0.43 b	N/D	N/D	N/D
2,3,5-Trimethylpyrazine[C]											
M.glyoxal	7.93 ± 1.18 a	22.16 ± 6.59 b	49.42 ± 2.49 c	14.42 ± 3.11 a	70.79 ± 7.71 dc	15.96 ± 0.35 a	101.93 ± 5.20 e	22.84 ± 8.19 b	55.71 ± 9.74 c	N/D	N/D
Pent.-2,3-dione	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
3-Ethyl-2,5-dimethylpyrazine[B]											
M.glyoxal	4.95 ± 1.35 ba	36.36 ± 11.02 dc	0.99 ± 0.03 a	18.88 ± 3.84 b	1.39 ± 0.10 a	10.48 ± 1.56 ba	2.85 ± 0.05 a	19.62 ± 5.59 b	2.69 ± 0.13 a	47.37 ± 4.39 c	29.27 ± 2.47 d
Pent.-2,3-dione	4.57 ± 0.62 a	5.34 ± 0.42 a	0.82 ± 0.06 b	9.28 ± 0.58 ca	1.29 ± 0.10 ba	7.77 ± 0.21 a	1.74 ± 0.21 ba	7.50 ± 0.58 a	1.04 ± 0.21 b	30.82 ± 11.68 d	38.71 ± 1.45 d
2-Ethyl-3,5-dimethylpyrazine[C]											
M.glyoxal	N/D	N/D	0.55 ± 0.02 a	N/D	0.78 ± 0.01 b	N/D	N/D	N/D	N/D	N/D	N/D
Pent.-2,3-dione	N/D	N/D	0.91 ± 0.11 a	N/D	1.42 ± 0.07 ba	2.68 ± 0.06 d	1.80 ± 0.16 b	2.07 ± 0.34 cb	1.22 ± 0.29 a	N/D	N/D
2,5-Diethyl-3,6-dimethylpyrazine[C]											
M.glyoxal	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
Pent.-2,3-dione	N/D	1.70 ± 0.51 b	0.87 ± 0.01 a	0.94 ± 0.04 ba	N/D	0.35 ± 0.01 a	N/D	1.67 ± 0.60 b	N/D	N/D	N/D
2,6-Diethyl-3,5-dimethylpyrazine[C]											
M.glyoxal	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
Pent.-2,3-dione	N/D	0.84 ± 0.25 b	0.14 ± 0.02 a	0.57 ± 0.03 ba	0.15 ± 0.05 a	0.22 ± 0.01 a	N/D	0.88 ± 0.30 b	0.12 ± 0.07 a	N/D	N/D
2,5-Dimethyl-3-(3-methylbutyl)pyrazine[C]											
M.glyoxal	N/D	2.01 ± 0.77 a	3.02 ± 0.61 a	6.43 ± 1.51 ba	5.63 ± 1.87 ba	4.44 ± 0.74 ba	5.90 ± 0.41 ba	7.92 ± 1.88 ba	12.01 ± 3.11 b	38.62 ± 0.90 c	36.59 ± 4.69 c
Pent.-2,3-dione	N/D	N/D	N/D	2.24 ± 0.28 a	N/D	2.30 ± 0.11 a	N/D	2.91 ± 0.16 a	N/D	31.70 ± 7.06 b	41.82 ± 2.94 c
Unreacted pentane-2,3-dione[C]											
Pent.-2,3-dione	N/D	N/D	77.09 ± 1.47 a	N/D	62.76 ± 4.82 b	N/D	58.01 ± 3.09 b	N/D	81.28 ± 2.15 a	N/D	N/D

[A] Data points represent mean values of three independent determinations and are given as GC/MS peak area x 10^6. Values in the same row followed by different letters are significantly different ($p<0.05$). Model reactions were as follows: dry mixtures of different whey hydrolysates + methylglyoxal and pentane-2,3-dione, free amino acids obtained from different whey hydrolysates as described in **Table 17** + methylglyoxal and pentane-2,3-dione, whey protein + methylglyoxal and pentane-2,3-dione. [B] Identification confirmed by GC retention index and mass spectra of authentic compounds. [C] Tentatively identified by matching mass spectra library. N/D, not detected

Another fact that explains why 2,5(6)-dimethylpyrazine was more abundant in the hydrolysate of proteinase from *Aspergillus melleus* than in the other hydrolysates is that this hydrolysate contains more dipeptides than other hydrolysates. Dipeptides were suggested to generate more pyrazines than free amino acids. Van Lancker et al. prepared Maillard model systems with lysine-containing dipeptides and glucose. It was found that, in most of the cases, dipeptides generated higher amounts of pyrazines than equimolar quantities of the free amino acid which are part of the dipeptide.[120] Additionally, other hypotheses that explain this phenomenon were discussed in **Section 3.3.3**. Furthermore, the whey protein hydrolysate from proteinase from *Aspergillus melleus* contains high levels of molecules with a molecular weight below 280 Da suggesting a considerable presence of dipeptides as well as free amino acids (**Figure 11**).

3-Ethyl-2,5-dimethylpyrazine was found in significant quantities in all model systems between hydrolysates and methylglyoxal. This is not surprising since it has been shown that it can have a different mechanism of formation, as described before. At the same time, it is known that threonine can generate 1-aminopropan-2-one by decarboxylation and dehydration reactions without the intervention of any dicarbonyl compounds. This α-amino ketone can further condense with 3-aminopentan-2-one, which is the resulting α-amino ketone obtained from the reaction of pentane-2,3-dione with amino acids or peptides, and form 3-ethyl-2,5-dimethylpyrazine as well. This is consistent with the results presented in **Table 19**, which shows that higher amounts of this pyrazine were found in model systems of whey protein hydrolysate from *Aspergillus melleus* and their corresponding amino acid mixture.

It was very interesting to find high amounts of 3-ethyl-2,5-dimethylpyrazine in a model system containing hydrolyzed whey from chymotrypsin and methylglyoxal. As mentioned before, several mechanisms are responsible for the formation of this compound (**Scheme 15**). However, none of the previously proposed mechanisms seems sound. **Figure 12** shows briefly other possibilities for the formation of 3-ethyl-2,5-dimethylpyrazine in this particular model system.

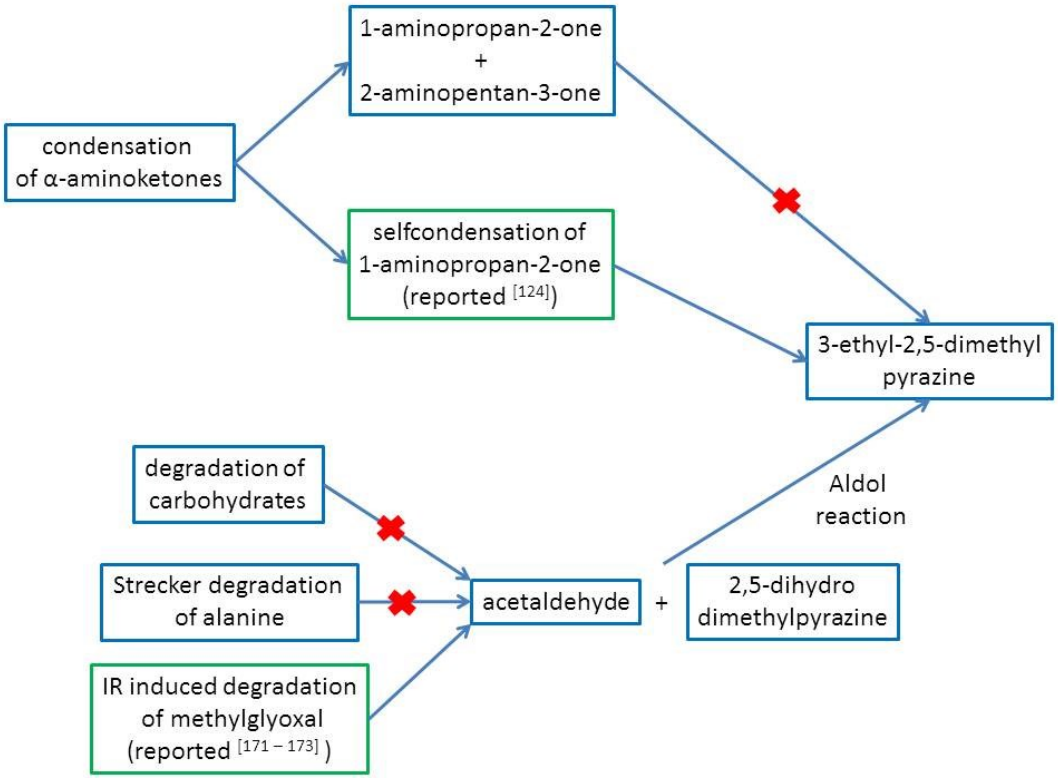


Figure 12. Degradation mechanismis leading to the formation of 3-ethyl-2,5-dimethylpyrazine in a model system containing methylglyoxal and whey protein hydrolysate obtained with chymotrypsin.

The pyrazine formation pathway from the condensation of 2,5-dimethyldihydropyrazines and acetaldehyde is considered as less favorable because acetaldehyde is either produced from the Strecker degradation of alanine, or from the degradation of carbohydrates. In this model system, alanine is present in small amounts, and at the same time these model systems do not contain any carbohydrates. Another formation pathway of this pyrazine is the condensation of 1-aminopropan-2-one and 2-aminopentan-3-one (obtained from the reaction of methylglyoxal and pentane-2,3-dione with amino acids or peptides, respectively). This mechanism does not seem possible since this model system does not contain pentane-2,3-dione. Therefore, it can be concluded that other mechanisms might be operative for the formation of this pyrazine. Rizzi et al. suggested that 3-ethyl-2,5-dimethylpyrazine can be formed via thermally induced autocondensation of 1-amino-propan-2-one derived from the reaction of methylglyoxal with the *N*-terminal amino acids of the peptides.[124] Moreover, 1-amino-propan-2-one has been shown to produce 2,5-dimethylpyrazine and trimethylpyrazine as well.[124] Additionally, It has been proposed that methylglyoxal can decompose into acetaldehyde and carbon monoxide via a decarbonylation reaction under the influence of infrared radiation.[171-173] In our model systems, the infrared radiation could be provided by the thermal conditions of the experiment. Moreover, the additional formation of acetaldehyde and its

further reaction with 2,5-dimethyldihydropyrazine would generate the missing ethyl substituent in the pyrazine ring. These last two mechanisms seem to be logic, since 3-ethyl-2,5-dimethylpyrazine was found in all model systems containing methylglyoxal.

In contrast as in the hydrolysates containing model systems, 3-ethyl-2,5-dimethylpyrazine was detected in small quantities in the model system containing the amino acid mixture obtained upon chymotrypsin, thermolysin or peptic digestion and methylglyoxal. However, this is most likely because the hydrolysates contained more amino sources to react with the dicarbonyl compound. The model system containing the amino acid mixture obtained upon hydrolysis with proteinase from *Aspergillus melleus* and methylglyoxal generated of a higher level of this pyrazine. Nevertheless, it may be due to the presence of a high amount of alanine and serine.

Trimethylpyrazine was detected only in model systems containing methylglyoxal, except in the case of proteinase from *Aspergillus melleus*, in which it was not reported due to an overlap with other substances eluting at the same retention time. It is important to mention that this particular pyrazine was produced in higher quantities from the free amino acid model system of chymotrypsin and thermolysine, than from the corresponding whey hydrolysates. Meanwhile, the opposite trend was observed for peptic hydrolysates and their corresponding free amino acid model systems. Additionally, it is possible that in all the model systems between the different hydrolysates and glucose trimethylpyrazine was also not detected (**Table 18**, **Table 19**) most likely due to overlapping between the isomers of 2-ethyl-(3)(5)(6)-methylpyrazine.

Finally, 2,5-dimethyl-3-(3-methylbutyl)pyrazine was found in similar amounts when comparing the different hydrolysates with their respective amino acid mixtures. However, it was produced in higher amounts in the model systems containing the hydrolysate from *Aspergillus melleus* or its corresponding amino acid mixture.

The set of experiments, which were made in model systems containing α-diones, were initially planned in order to elucidate how different pyrazines can be generated. It was expected that if the α-diones were limited, the formation of pyrazines would be limited and could be predicted. Therefore, when pyrazines exhibits a specific substituent, this experimental design would allow identifying its origin. Nevertheless, the results depicted in this section have shown that other pyrazines were produced as well, suggesting that the mechanisms leading to the formation of pyrazines are far from being simple.

4.4 CONCLUSIONS

The results depicted in this chapter offer a systematic comparison of the generation of pyrazines in various models of native whey protein isolate and five different whey protein hydrolysates in which amino acids and peptides are simultaneously present. The influence of conditions such as reactant ratios and type of reactant was found to be critical. The results suggest that in most of the cases the role of peptides in pyrazine formation was significantly higher in comparison with free amino acid, even in the model systems where the amount of amino acids was considerably high. This confirms the role of peptides as an important flavor precursor.

Finally, this study illustrates clearly the complexity of the mechanism leading to the formation of pyrazines in Maillard model systems, this includes:

1. Formation of different aminoketones from the reaction of amino acids and dicarbonyl compounds (**Scheme 5**, **Section 1.7.1**)
2. Aldol reactions between Strecker aldehydes generated from amino acids and a dihydropyrazine anion (**Scheme 5 II**, **Scheme 6**, **Section 1.7.1**)
3. Formation of aminoketones from pyrolysis of serine and threonine (**Scheme 8, Scheme 9, Section 1.7.2**)
4. Formation of different aminoketones from the reaction of peptides and dicarbonyl compounds (**Scheme 10**, **Section 1.7.3**)
5. Aldol reactions between aldehydes generated from from infrared degradation of dicarbonyl compounds and a dihydropyrazine anion (**Section 4.3.4**).

By taking in account all these possibilities, it is likely that in complex model systems or even in real food systems, the formation of pyrazines has not one unique origin. This suggests that some pyrazines are amino acid or peptide specific, while others can have multiple and synergistic formation mechanisms.

As in **Chapters 2**, **3** and **4**, it was demonstrated that peptides are effective pyrazine precursors, the following chapter evaluated if peptides can generate pyrazine in real food systems.

CHAPTER 5

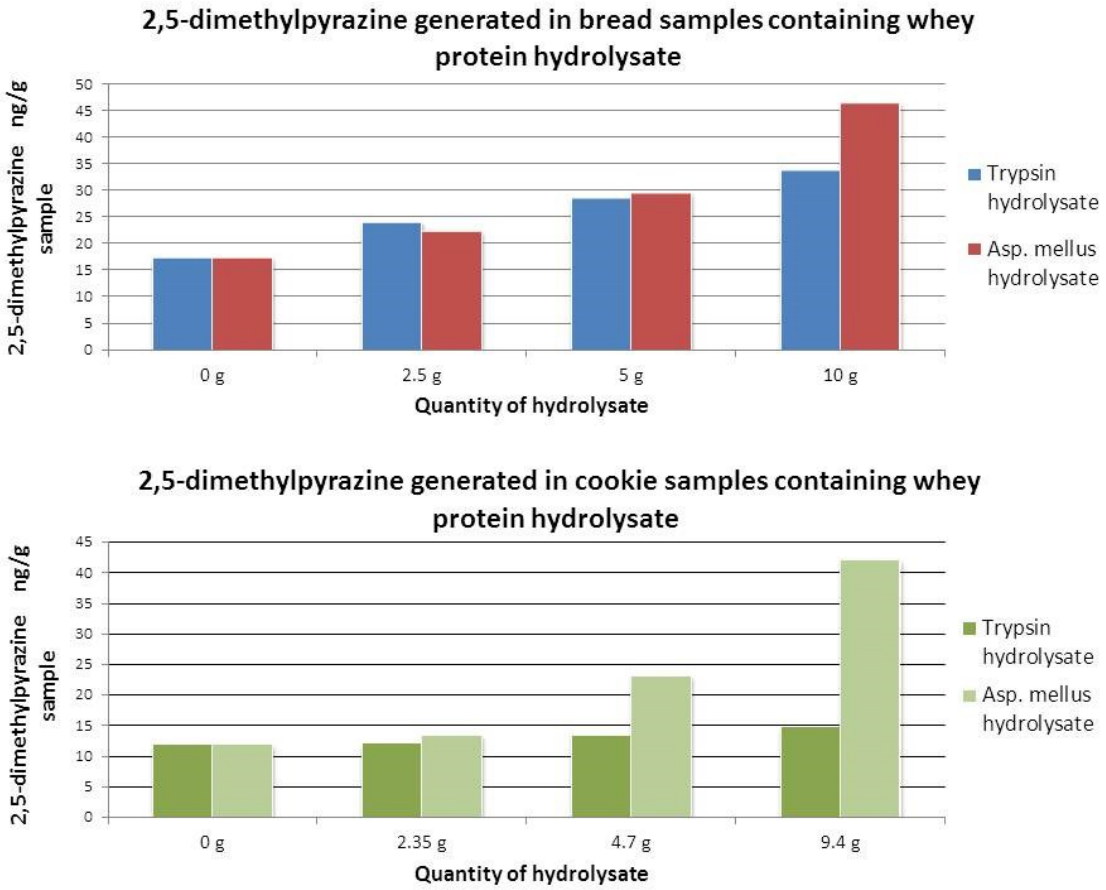
Impact of whey protein hydrolysates on the formation of 2,5-dimethylpyrazine in baked food products

ABSTRACT

Most investigations concerning the generation of pyrazines during the Maillard reaction were studied in different model systems varying in complexity. The information that can be found in the literature regarding alkylpyrazines in food products mainly reports the abundance and flavor characteristics of these compounds. The generation of pyrazines in real food systems is less investigated.

The present study investigated the impact of adding protein hydrolysates in baked food recipes, more specifically their potential as precursors for generation of 2,5-dimethylpyrazine in the obtained food products. It was found that the two different protein hydrolysates were effective on generating 2,5-dimethylpyrazine in two well-known food products, bread and cookies. A sensory panel further evaluated the overall aroma production in the samples, and as a result, it was found that the use of protein hydrolysates in baked food products had a remarkable impact in the aroma and general appearance of the baked food products.

KEYWORDS: peptides, whey protein, hydrolysates, Maillard reaction, pyrazines, HS-SPME-GC/MS, GPLC.



Graphic Abstract 4. Summarized information of **Chapter 5**

5.1 INTRODUCTION

The generation of flavor in the Maillard reaction has been the subject of numerous reviews and publications.[31,62,68,92,139,174-175] The Maillard reaction is influenced by temperature, time, pH, a_w, nature and proportion of the reactants. Furthermore, the generation of flavor using precursors has been studied as well. Flavor precursors are compounds which are naturally present or intentionally added to a formula to obtain a desired specific flavor. Methionine, for example, leads to the production of a "vegetable character".[176] Glucose and fructose have been reported to be suitable for increasing the roast character of flavors developing model systems.[176] Protein hydrolysates and peptides in general have been recognized as important flavor enhancers and precursors of the Maillard reaction.[9,21-23,176-177] Moreover, their use in Maillard model systems was effective to generate alkylpyrazines among other volatiles.[9,120,178]

As shown in the literature, the factors that affect the Maillard reaction have been extensively studied. However, these studies have been done mainly in model systems. While such insight is invaluable, it does not solve immediately the problem to make predictions for what happens in real food. As for example according to Mottram et al.[68] model systems never deliver all the sensory characteristics of cooked food. van Boekel et al.[70] presented a SWOT analysis on the predictive modeling of flavor compound formation during the Maillard reactions. In spite of the fact that this work focused on kinetic modeling, the authors offered an interesting conclusion that it is applicable to non-kinetic models as well. The weak points in current modeling are that the translation from simplified model systems to real food systems is problematic. Moreover, the very diverse and conflicting results in literature show a large uncertainty on their application.

Several parameters need to be taken into account when considering the formation of flavor compounds in food products. Taylor et al.[179] described in detail how some physical properties of different food matrices such as the mobility of the reactants, the temperature profile in different locations of matrix or phase changes caused by heating can affect the course of the Maillard reaction. As for example, in many cereal-based food where the initial matrix is a dough or a batter, the initial water content of the food matrices ranges between 20 – 50 % w/w before cooking. In these conditions, the matrix consists of a mixture of food components. This matrix can undergo dehydration while being thermally treated, influencing the Maillard reaction through the concentration of some water-soluble solutes in the outer regions of the product. The authors provided other examples as well and for such reason they agree in the necessity to study the Maillard reaction in more suitable model systems.[179]

Other aspects that play an important role in the formation of volatiles in food products are the multiple interactions between the different ingredients. The aroma of most foods that are thermally

processed originates mainly from the Maillard reaction and in part from lipid autoxidation products.[180] Nevertheless, there is plenty evidence that the products of both reactions can interact to form compounds in the intermediate and final stages of the Maillard reaction.[181-182] Moreover, Zamora and Hidalgo reports that the existing data suggest that both reactions are so interrelated that they should be considered simultaneously when it is intended to describe the formation of products from the Maillard reaction in the presence of lipids and vice versa.[182] As for example, Hidalgo and Zamora reported the formation of phenylacetaldehyde (Strecker aldehyde of phenylalanine) in Strecker-type degradation reactions initiated by 4,5-epoxy-alk-2-enals (secondary products of lipid peroxidation).[183]

The generation of pyrazines due to the Maillard reaction was studied extensively. However, to the best of my knowledge, information regarding the influence of lipids on the formation of these particular volatiles seems to be limited. Negroni et al.[180] studied the effects of different oils on the formation of volatiles from the Maillard reaction in model systems of lysine – xylose and lysine – glucose. The authors found that the production of pyrazines appeared to be particularly sensitive to the degree of unsaturation of the oils. In both lysine – xylose and lysine – glucose model systems, the unsubstituted pyrazine was formed more with olive oil, while for canola oil and sunflower oil, it was produced less. On the contrary, 2,5-dimethylpyrazine, and 2,3-dimethylpyrazine were formed less with olive oil, while the generation of these pyrazines increased in models containing canola oil and sunflower oil. Additionally, the formation of 2-methylpyrazine was found to be variable depending on the model system (lysine – xylose and lysine – glucose). Its formation was promoted in lysine – xylose models containing canola oil, while in lysine – glucose models, the addition of canola oil hindered the formation of 2-methylpyrazine.[180] The authors reported that the presence of oils had a direct impact on the formation of pyrazines. However, a satisfactory explanation of this phenomenon could not be given.

The conclusions, presented by van Boekel et al.,[70] Taylor et al.[179] and Mottram et al.[68] emphasize the relevance of formulating more complex model systems or food-like model systems to conduct detailed studies on Maillard chemistry in food.

Low et al.[167] used model potato cakes to study the formation of alkylpyrazines in systems containing added glycine, which is used by the food industry to limit the formation of acrylamide. Madruga et al.[59,135] determined some aroma precursors in goat meat and their role in the flavor formation during cooking. Balagiannis et al.[184-185] published semi-empirical models using aqueous liver and muscle extracts. The authors were able to predict the formation of Strecker aldehydes **36**, although the generation of pyrazines was also monitored. More recently, Zheng et al.[186] developed a quite adequate canned-coffee model system to study the formation of furan and other Maillard products

like 2-methylbutanal and 2,6-dimethylpyrazine. However, to the best of our knowledge, the generation of pyrazines in food products as a result of the addition of specific flavor precursors during the Maillard reaction has been less studied.

Peptides have been used in different Maillard model systems (**Chapters 2 – 4**). This study aimed to evaluate the effect of peptides, as flavor precursors of alkylpyrazines during the Maillard reaction in real food systems and processing conditions. Moreover, the generation of alkylpyrazines was followed using a labeled internal standard.

Peptides can be obtained upon controlled enzymatic hydrolysis of proteins which represents an advantage for the industry as the process can be standardized (**Section 1.2**, **Section 1.3**). The addition of peptides seems suitable to be used as a flavor precursor for baked or roasted products in general, aiming to generate characteristic roasted aromas in food products, without the necessity of adding artificial flavors.

5.2 MATERIALS AND METHODS

5.2.1 Chemicals

D-(+)-glucose (99.5%), trypsin from porcine pancreas and proteinase from *Aspergillus melleus,* were purchased from Sigma–Aldrich (Bornem, Belgium). 2,5-dimethylpyrazine (99%), was purchased from Acros Organics, Thermo – Fisher Scientific (Erembodegem, Belgium). Whey protein isolate LACPRODAN DI-9224 was donated from Arla foods (Aarhus, Denmark). A deuterated standard, consisting of a mixture of 2-[$^{2}H_{3}$]-methyl-3-methylpyrazine (~83%) and 2-[$^{2}H_{3}$]-methyl-6-methylpyrazine (~17%), was synthetized[187] and generously donated by Prof. Keith Cadwallader (Department of Food Science and Human Nutrition, University of Illinois, United States of America).

Wheat flour (15.5 % moisture), eggs (chicken), sugar (sucrose), dried baker's yeast, salt, butter, sodium bicarbonate and baking paper were purchased in a local store.

5.2.2 Hydrolysis of whey protein

Two whey protein hydrolysates were considered for this set of experiments: tryptic whey protein hydrolysate (**Section 2.2.2**) and whey protein hydrolysate obtained upon enzymatic digestion with proteinase from *Aspergillus melleus* (**Section 4.2.2**). The tryptic hydrolysate was selected since it was successfully used to study Maillard model systems under a variety of conditions described in **Chapter 2**. Whey protein hydrolysate obtained with proteinase from *Aspergillus melleus* was selected

because it was characterized as a quite different hydrolysate as compared to other studied hydrolysates (**Section 4.3.1**).

Both hydrolysates were obtained in sufficient quantities to be added to the food recipes. Since these enzymatic hydrolysis were done in regular basis, the amino acid and peptide composition of the hydrolysates is linked to **Section 2.2.4** and **Section 4.3.1**.

5.2.3 Preparation of food matrices

Two food products, cookies and bread were selected for the presented work, as they represent different formulations and manufacturing process.

Whey protein isolate and hydrolyzed whey protein were added as ingredients in quantities which corresponds to 9.40 g for the cookie dough and 10 g for the bread dough, respectively. The quantities of whey protein and hydrolyzed whey protein were added in different proportions ranging from 0 to 100 % over the total amount present in the food formulation. The formulation of the different cookies and bread recipes is presented in **Table 21**.

Table 21. Quantities of whey protein and whey protein hydrolysate present in the different food products.

	Name of the sample	Whey protein isolate content	Tryptic hydrolysate content	Hydrolysate from *A. melleus* proteinase content
Food product				
Cookies	C1	9.40 g	0 g	0 g
	C2	7.05 g	2.35 g	0 g
	C3	4.7 g	4.7 g	0 g
	C4	0 g	9.40 g	0 g
	C5	7.05 g	0 g	2.35 g
	C6	4.7 g	0 g	4.7 g
	C7	0 g	0 g	9.40 g
Bread	B1	10 g	0 g	0 g
	B2	7.5 g	2.5 g	0 g
	B3	5 g	5 g	0 g
	B4	0 g	10 g	0 g
	B5	7.5 g	0 g	2.5
	B6	5 g	0 g	5 g
	B7	0 g	0 g	10 g

5.2.3.1 Preparation of cookie samples

The cookies were prepared following the AACC method 10-50D (American Association of Cereal Chemists, 2000). Additionally, the whey protein and/or hydrolyzed whey protein was added in quantities comprising the 4% of the original recipe, this is 9.40 g.

32 g of soft butter, 60 g of sucrose, 6.0 g of glucose, 1.0 g of salt, 1.25 g of sodium bicarbonate and 9.40 g of whey protein and / or hydrolyzed whey protein in different proportions (**Table 21**) were combined in a bowl and mixed with an automatic kitchen mixer (Kenwood) for three minutes at low speed. 21.5 g of water were added and the matrix was mixed for one minute at high speed, 114.5 g of wheat flour (15.5% moisture content) were added and mixed again at low speed for two minutes. The resulting dough was rolled out until 1 cm thickness was obtained and then cut into several cookies using a round cookie cutter (5.0 cm of diameter). Finally, the cookies were placed on a baking sheet previously covered with parchment paper and then baked at 205 °C for 10 minutes in an oven (Memmert, Fisher Scientific, Erembodegem, Belgium.) (**Figure 13**). The percentages of the different ingredients in the cookie samples are detailed in **Table 22**.

Table 22. Cookie samples formulation

Ingredients	quantities (g)	Percentage (%)
Butter	32	13.09
Sucrose	60	25.54
Glucose	6	2.45
Salt	0.5	0.20
Sodium bicarbonate	0.62	0.25
water	21.5	8.79
wheat flour	114.5	46.83
Added whey protein / hydrolysate	9.40	3.84
Total	244.52	100

Figure 13. Cookies obtained following the procedure detailed in **Section 5.2.3.1**

5.2.3.2 Preparation of bread samples

The bread was prepared by following a modified recipe of a traditional cooking book, in which the milk content was replaced by 10.0 g of whey protein and/or hydrolyzed whey protein and water. Initially, 20.0 g of dry yeast powder and 10.0 g of whey protein isolate and / or hydrolysate (**Table 23**) were mixed with 50 ml water. The resulting solution was then manually mixed with 50 g of wheat flour and then left to rest for 15 minutes. Afterwards, 150 g of wheat flour, one egg, 15.0 g of sucrose and 5.0 g of glucose were added to the mixture and kneaded manually until it reached an appropriate consistency. The resulting mixture was kneaded intensively for seven minutes. Subsequently, 40 g of soft butter was added and the mixture was kneaded until the butter was fully incorporated. 4.0 g of salt were added and the mixture was kneaded for an additional five minutes. The resulting dough was then left to rest under a plastic wrap for a total of 45 min. Meanwhile the dough was flattened, stretched and folded every 15 minutes. Finally, after 45 min, the dough was flattened and shaped into a firm ball on a baking sheet, then left to rest for 15 minutes (**Figure 14**) and baked in an oven at 200°C for 25 minutes (Memmert) (**Figures 15 – 17**). The percentages of the different ingredients in the bread samples are detailed in **Table 23**.

Table 23. Bread samples formulation

Ingredients	quantities (g)	percentage
Yeast powder	20	5.08
water	50	12.69
wheat flour	200	50.76
Eggs	50	12.69
Sucrose	15	3.81
Glucose	5	1.27
Butter	40	10.15
Salt	4	1.02
whey protein / hydrolysate	10	2.54
Total	394	100

Figure 14. Bread samples before baking

Figure 15. Bread samples after baking.

Figure 16. Detail of the crumb of a bread sample

Figure 17. Detail of the crumb of a bread containing *Asp. melleus* hydrolysate (**sample B7**)

5.2.4 HS-GC-MS Analysis

5.2.4.1 Preparation of the samples

All samples were ground separately in an automatic mixer (Kenwood) to assure sample homogeneity. For each of the obtained powders, 1.0 g was put in a 20.0 ml SPME vial (Gerstel, Mulheim, Germany). Further, 0.5 µl of deuterated standard, consisting of a mixture of 2-[$^2H_3$]-methyl-3-methylpyrazine (~83%) and 2-[$^2H_3$]-methyl-6-methylpyrazine (~17%) were added when necessary (**Section 5.2.4.2.2**) in a 0.05 mg/ml ethyl acetate solution with a 10 µl Hamilton syringe and the vial was immediately capped with a pressure cap [silicone/poly-(tetrafluoroethylene) (PTFE), 55^o, shore A, 1.5 mm, magnetic; Gerstel, Germany].

5.2.4.2 GC-MS conditions

5.2.4.2.1 Preliminary experiment.

There is an important loss of the volatiles from the cookies and bread samples during the baking process. Nevertheless, it was expected that some remaining flavor compounds would be still present

in the food matrices. Therefore, The SPME vials containing the food samples were pre-incubated at 70 ^{o}C for 3 minutes and further extracted by means of HS-SPME using a DVD/Car/PDMS fiber (Supelco, Bornem) with a multipurpose sampler (CombiPAL), (CTC Analytics, Agilent technologies). GC – MS analyses of the SPME extract were done with an Agilent 7890A GC Plus apparatus coupled to a quadrupole mass spectrometer 5975 MSD (Agilent Technologies, Diegem, Belgium) and equipped with an DB-624 capillary column (60 m length x 0.250 µm i.d; 1.4 µm film thickness) (Agilent Technologies, Diegem). Working conditions were: transfer line to MSD 280 ^{o}C, carrier gas (He) 1.0 ml/min; ionization: EI 70eV; acquisition parameters: Scanned m/z: 30-450; oven temperature started at 35 ^{o}C, held for 1 min, programmed from 35 – 250 ^{o}C at 3 ^{o}C/min and held 10 min. The volatiles were identified by comparison of the mass spectrum with mass spectral libraries (Nist 08, Wiley 6th and HPCH2205) and confirmed when possible by matching the mass spectra and retention time of the authentic compounds. The generation of the different volatiles was followed in a semiquantitative way by considering the absolute peak area of each individual compound in the chromatogram (**Section 2.2.8**).

5.2.4.2.2 2,5-Dimethylpyrazine analysis.

The quantification of 2,5-dimethylpyrazine was achieved by using an internal standard. In order to avoid absorption issues using a SPME method, the volatiles were extracted using headspace (HS).
The SPME vials containing the food samples were incubated at 70 ^{o}C for 15 minutes in order to release the flavor compounds to the headspace of the vial. Further, The different samples were extracted by means of headspace (HS) with a HD-type gas tight syringe of 2,5 ml (CTC Analytics, Agilent technologies, Zwingen, Switzerland) with a multipurpose sampler (CombiPAL), (CTC Analytics, Agilent technologies). GC – MS analyses of the HS extract were done with an Agilent 7890A GC Plus apparatus coupled to a quadrupole mass spectrometer 5975 MSD (Agilent Technologies, Diegem, Belgium) and equipped with an DB-624 capillary column (60 m length x 0.250 µm i.d; 1.4 µm film thickness) (Agilent Technologies, Diegem). Working conditions were: transfer line to MSD 280 ^{o}C, carrier gas (He) 1.0 ml/min; ionization: EI 70eV; acquisition parameters: SIM low resolution, monitored ions (108 m/z for 2,5-dimethylpyrazine and 111 m/z for 2-[$^{2}H_3$]-methyl-3-methylpyrazine and 2-[$^{2}H_3$]-methyl-6-methylpyrazine; oven temperature started at 35 ^{o}C, held for 1 min, programmed from 35 – 120 ^{o}C at 15 ^{o}C/min, then from 120 – 130 ^{o}C at 2 ^{o}C/min and held for 2 min, then from 130 – 150 ^{o}C at 2 ^{o}C/min and from 150 – 250 ^{o}C at 20 ^{o}C/min and held 5 min. 2,5-dimethylpyrazine was identified by comparison of the mass spectrum and retention time of the authentic compound.

The generation of 2,5-dimethylpyrazine was followed by quantification using an external calibration curve using the authentic compound (2,5-dimethylpyrazine) ranging from 0 to 100 ng and a mixture of 2-[$^{2}H_3$]-methyl-3-methylpyrazine (~83%) and 2-[$^{2}H_3$]-methyl-6-methylpyrazine (~17%) deuterated internal standard (500 ng).

5.2.5 Statistical analysis

The various cookie and bread samples were grinded resulting in seven different cookie and seven different bread powders. From each one of the batches, 2.0 g of powder were taken and placed in a 20.0 ml SPME vial, the rest of the batch was shaken. These steps were repeated two more times, obtaining then triplicates from which the data points of the HS-GC-MS analysis of 2,5-dimethylpyrazine were obtained. The data points were normally distributed (Kolmogorov-Smirnov test: ($p < 0.050$) for all standardized residuals) and represent mean values of three determinations. Therefore, one way ANOVA was selected for statistical analysis. The Tukey correction was applied to control the family-wise error rate at 5% for all multiple pairwise comparisons.

In the sensory analysis, Friedman's test and post hoc analysis were done at a significance level of $p < 0.05$. The Friedman test is the nonparametric equivalent to the two-way analysis of variance without interaction. The Friedman test equation is based on the $\chi 2$ distribution. All analyses were done using SPSS Statistics version 22.

5.2.6 Sensory analysis

A sensory analysis was made in order to identify the highest preference in aroma and appearance in cookies and bread samples respectively. General guidelines of the test were followed as described by Meilgaard et al.[188] Further, a preference-ranking test [189] was conducted examining two attributes, the overall appearance and the aroma. In these tests the panel members are asked to rank several products in either descending or ascending order of preference or liking. The participants are usually not allowed to have ties in the ranking, thus the method implies a forced choice. Preference ranking is intuitively simple for the consumer and it can be done quickly and with relatively little effort. The ranks are based on a frame of reference that is internal to the specific set of products and thus the consumer does not have to rely on memory. The data are ordinal and are treated as nonparametric.

The sensory evaluation of the bread and the cookies was done in the sensory facilities of the Faculty of Bioscience Engineering from Ghent University.

A group of 21 panelists of different ages, nationalities and gender, characterized as frequent consumers of bread and cookies was recruited and briefly instructed prior to the analysis. The

sensory test was carried out in two consecutive days, one for each product. During both days, three groups of seven people entered the sensory evaluation lab, further, four different samples were presented at room temperature (20–22 °C) in random order, labeled with randomly-generated three-digit codes. The bread and cookie samples were one day old.

Panelists were asked at first to evaluate the degree of preference towards the appearance of the samples and secondly to evaluate the preference towards the aroma under red light to prevent the visual appearance of the samples affecting the judgment of the panelists. For each sensory attribute a different set of samples was introduced. An example of the sensory analysis survey can be found in **Appendix 4.** The score sheets required from the panelists to rank four samples in the order of preference from 1-4 with: (1) least preferred, (2) for the next, (3) for the next, (4) for the most preferred. In addition, they were requested to comment on their choices. Moreover, the sum of the rank values can be used to determine which samples are the most preferred. Nevertheless, a statistical treatment of the data is often necessary. Friedman's test ($p < 0.05$), was used to determine whether significant differences were present among the samples. Least significant rank difference test (LSRD) (**eq. 1**) was used to determine which samples were different. Two samples differ significantly at an $\alpha = 0.05$ if their rank sums differ more than the value of LSRD

$$LSRD = t_{\alpha/2,\infty}\frac{\sqrt{JK(J+1)}}{6} \qquad \textbf{(eq. 1)}$$

Were J is the number of samples, K is the number of panelists and $t_{\alpha/2,\infty}$ is the upper- $\alpha/2$ critical value of a t-distribution for infinite degrees of freedom (Meilgaard et al. 2006).

5.3 RESULTS AND DISCUSSION

5.3.1 Selection of the volatiles to monitor

The work previously presented in **Chapter 2**, **Chapter 3** and **Chapter 4** suggested that peptides are important precursors of alkylpyrazines.[9,120-121,129,139,178] Therefore, a preliminary experiment was made in order to determine which alkylpyrazine among other volatiles was more abundant in two different cookies samples (**Table 24**). One cookie contained 9.40 g of tryptic hydrolyzed whey protein (**Section 2.2.2**) and the other 9.40 g of hydrolyzed whey protein obtained with proteinase from *Aspergillus melleus* (**Section 4.2.2**). The detection of the volatiles was evaluated by solid phase micro extraction coupled with gas chromatography and mass spectrometry (HS-SPME-GC/MS) using the method described in **Section 5.2.4.2.1**.

Several volatiles compounds were detected in the cookie samples, including pyrazines. However, the identification of the volatile compounds was only possible in some cases, as presented in **Table 24**. Moreover, not all the reported substances are generated due to the Maillard reaction.

Hexenal, 2-heptanone, heptanal, octanal and heptanoic acid are formed as consequence of lipid oxidation of fatty acids.[190] Additionally, Shi et al.[191] described the formation of limonene in oxidized tallow, and in Maillard model systems containing oxidized tallow. Still, there is little information of the formation mechanisms of this compound upon the Maillard reaction or lipid oxidation reactions. Other volatiles such as 3-methylbutanal, 2-methylbutanal, methional and phenylacetaldehyde are Strecker aldehydes generated due to the Maillard reaction. Further, methional can degrade to methanethiol and further oxidize to form dimethyl disulfide.

Pyridines, are formed mainly during the thermal degradation of sulfur-containing amino acids alone or in the presence of glucose.[8] They are also formed in glucose–proline systems, on degradation of Amadori intermediates of the glucose–glycine system, and on pyrolysis of α- and β-alanine.[8] Moreover, pyridines and other nitrogen-containing heterocycles have been shown to be present in melanoidins (products of advanced Maillard reactions).[8]

It is clear that all the different flavor compounds present in a food product will have an impact in its organoleptic properties. Nevertheless, the main objective of this set of experiments is to investigate the formation of alkylpyrazines as consequence of peptide addition in food matrices. As shown in **Table 24**, 2,5-dimethylpyrazine was present in all the samples, while 2-ethylpyrazine, 2,3-dimethylpyrazine, 2-ethyl-6-methylpyrazine, 2-ethyl-3-methylpyrazine and 3-ethyl-2,5-dimethylpyrazine were only present in the cookies containing hydrolysate from the *A .melleus* hydrolysate. As 2,5-dimethylpyrazine was present in all the different samples, it was selected to estimate the overall impact of the whey hydrolysate addition in the Maillard reaction in the selected food samples. Moreover, this compound was reported extensively in Maillard model systems for a large variety of conditions and products.[7,92]

Table 24. Overall identified volatiles in two different cookie formulations.

Compound [A]	Cookies with non-hydrolyzed whey	Cookies with *Asp. melleus* hydrolysate	Cookies with tryptic hydrolysate	Odor description [C]
	GC/MS peak area x 10^6			
3-Methylbutanal [A]	N/D	40.01	0.30	Chocolate, fruitty, fatty
2-Methylbutanal [B]	N/D	28.68	N/D	Musty, chocolate, nutty, malty
Dimethyl disulfide [B]	N/D	0.40	N/D	Sulfurous, vegetable, cabbage, onion
Pyridine [A]	376.35	247.07	353.38	Sour, fishy, ammonia
Hexenal [B]	5.03	3.52	3.21	Vegetative, fatty, fruity with a woody nuance
Furfural [B]	2.19	0.85	0.49	Sweet, woody, baked bread, caramel
2-Heptanone [B]	2.99	5.30	4.01	Fruity, spicy, sweet, herbal, coconut, woody
Heptanal [B]	1.29	1.07	0.96	Fresh, fatty, herbal, wine
2,5-Dimethylpyrazine [A]	0.26	12.73	3.37	Cocoa, roasted, nuts, roast beef, woody
2-Ethylpyrazine [A]	N/D	0.18	N/D	Peanut, musty, nutty, woody, roasted cocoa
2,3-Dimethylpyrazine [A]	N/D	0.23	N/D	Nutty, coffee, peanut butter, walnut, caramel
Methional [A]	N/D	1.16	N/D	Musty, potato, mold ripened cheeses, onion
2-Ethyl-6-methylpyrazine [B]	N/D	0.57	N/D	Roasted hazelnut, nutty
2-Ethyl-3-methylpyrazine [A]	N/D	2.93	N/D	Nutty, peanut, musty, earthy, bread
Octanal [B]	1.19	0.76	0.65	Waxy, citrus, orange
Limonene [A]	6.85	3.94	7.86	Herbal, peppery
3-ethyl-2,5-dimethylpyrazine [A]	N/D	0.55	N/D	Nutty
Phenylacetaldehyde [A]	N/D	1.98	N/D	Grass, sweet, floral, clover, cocoa
Heptanoic acid [B]	15.84	9.38	9.31	Cheesy, waxy, fermented, pineapple
3,5-Dihydroxy-6-methyl-2,3-dihydropyran-4-one [B]	5.79	27.31	8.74	Non-described
2-Undecanone [B]	2.08	1.74	1.04	Waxy, fruity, fatty

[A] Identification confirmed by GC retention index and mass spectra of authentic compounds. [B] Identification based in the best spectra match available in the library. [C] Odor descriptors obtained from http://www.thegoodscentscompany.com/

5.3.2 Characterization of the hydrolysates in peptides and amino acids

Two whey protein hydrolysates were selected to be used as part of the formulation of bread and cookies. One hydrolysate was obtained from the digestion of whey protein with trypsin while the other was obtained using proteinase from *Aspergillus melleus*. These two hydrolysates have been

previously characterized in terms of their free amino acid composition and their approximate peptide profile (**Section 2.2.3, Section 2.2.4** and **Section 4.3.1)**.

As it was be observed in previous chapters both hydrolysates were significantly different in their peptidic profile and free amino acid composition. In the case of the tryptic hydrolysate, the quantities of free amino acids are significantly lower compared to the hydrolysate obtained with proteinase from *Aspergillus melleus* (**Table 17**). At the same time, it can be seen that the peptidic profile between the hydrolysates was also different (**Figure 4A** and **Figure 11**). The hydrolysate obtained using the proteinase from *A. melleus* contained a high amount of peptides with molecular weights below 1355 Da, while in the tryptic hydrolysate the majority of the peptides had a molecular weight between 1355 and 8000 Da. Hence, it was expected that the flavor formation from these hydrolysates would be different as well.

5.3.3 Formation of 2,5-dimethylpyrazine

The results presented in **Table 25** show that for all cases, the use of whey protein hydrolysates resulted in an enhanced production of 2,5-dimethylpyrazine with respect to the control samples (C1 and B1). In both food products, the samples containing tryptic hydrolysate exhibited a moderate rise in the detected amounts of 2,5-dimethylpyrazine. This increment of the 2,5-dimethylpyrazine quantities was found to be proportional to the concentration of tryptic hydrolysate in the samples. However, this trend was considerably more pronounced in the case of the food samples containing the hydrolysate from *A. melleus* proteinase. This observation can be due to the fact that the tryptic hydrolysate contains small amounts of free amino acids and rather medium molecular weight peptides. On the other hand, the hydrolysate from *A. melleus* proteinase has a considerable higher amount of free amino acids and abundance of small peptides (**Table 16**, **Table 17**). Additionally, the use of both whey protein hydrolysates generated statistically similar quantities of 2,5-dimethylpyrazine for cookies as well as for bread samples when the amount of hydrolysate corresponded to 2.35 g for cookies and 2.5 g for bread (samples C2, C5, B2 and B5). Samples B3 and B6, showed that the production of 2,5-dimethylpyrazine was similar in bread samples. However, the use of hydrolysate from *A. melleus* resulted in an enhanced generation of 2,5-dimethylpyrazine in the cookie samples (samples C3 and C6). In samples C4, C7, B4 and B7, the generation of 2,5-dimethylpyrazine was clearly favored when the hydrolysate from *A. melleus* was used in both food products.

Table 25. 2,5-Dimethylpyrazine* detected in bread and cookie samples. Quantities are expressed in ng/g of sample.

Amount of whey protein hydrolysate	0 g	2.5 g	5 g	10 g	2.5 g	5 g	10 g
Sample (bread)	B1	B2	B3	B4	B5	B6	B7
2,5-Dimethylpyrazine	17.29 ± 0.88 a	24.01 ± 1.28 b	28.44 ± 2.26 c	33.80 ± 2.41 d	22.21 ± 0.68 ab	29.47 ± 1.60 dc	46.25 ± 2.85 e
Amount of whey protein hydrolysate	0 g	2.35 g	4.7 g	9.4 g	2.35 g	4.7 g	9.4 g
Sample (cookies)	C1	C2	C3	C4	C5	C6	C7
2,5-Dimethylpyrazine	11.78 ± 0.76 a	12.02 ± 0.38 a	13.30 ± 1.10 ab	14.66 ± 0.28 b	13.26 ± 0.15 ab	22.99 ± 0.91 c	41.99 ± 1.73 d

* Identification confirmed by GC retention index and mass spectra of the authentic compound. Data points represent mean values of three independent determinations. Values in the same row followed by different letters are significantly different ($p<0.05$).

It is also interesting to mention that in general, more 2,5-dimethylpyrazine was found in bread samples than cookie samples. This fact is most likely related to the addition of yeast during the bread manufacturing. Munch et al.[192-193] reported that yeast has a high content of free ornithine. The Strecker degradation of this amino acid generates 4-amino-butanal, which further cyclizes to form 1-pyrroline. 1-Pyrroline is a precursor of 2-acetyl-1-pyrroline and 2-acetyltetrahydropyridine, two key odorants of bread. However, as a result of the Strecker degradation, ornithine generates α-aminoketones as well. These α-aminoketones can condense to form pyrazines (**Section 1.7.1**).

Additionally, bread samples were heated longer than the cookie samples. During the baking process, the bread crust is exposed to a higher temperature than the crumb. As the bread matrix dehydrates, it concentrates some water-soluble solutes in the outer regions of the product.[179] Since the presence of pyrazines in the bread crust was reported previously[194-195], it is likely that the combination of the mentioned factors influenced the formation of 2,5-dimethylpyrazine. Additionally, Negroni et al.[180] reported that different oils can promote the formation of pyrazines in Maillard model systems depending on their unsaturation degree. Butter has unsaturated fatty acids as part of its composition (**Table 26**). Nevertheless, bread and cookie samples contained butter in similar percentages (**Table 22**, **Table 23**).

Table 26. Fat composition of average butter samples. Values obtained from The 'Composition of Foods Integrated Dataset' (CoFID), National Diet and Nutrition Survey, Public Health England (PHE).

	Fat	Saturated fatty acids	n-6 polyunsaturated fatty acids	n-3 polyunsaturated fatty acids	cis-Monounsaturated fatty acids	Monounsaturated fatty acids	cis-Polyunsaturated fatty acids	Polyunsaturated fatty acids	Trans fatty acids
Quantities are expressed in g/100 g food									
Butter Average	82.2	52.09	1.41	0.68	18.48	20.91	2.27	2.83	2.87

5.3.4 Sensory analysis

Friedman's test report suggests that significant differences exist among the samples if the calculated test value exceeds the upper-α critical value of a chi-square random variable for (t-1) degrees of freedom (Meilgaard et al. 2006). Hence, for our case a significant difference is present, for each attribute examined among the samples, if the chi-square is greater than $\chi^2(3) = 7.81$ for $p= 0.05$. Furthermore, two samples are characterized to be significantly different ($\alpha= 0.05$) if their rank sums difference is greater than LSRD= 16.4 from (**eq. 1**).

The use of a tryptic whey protein hydrolysate in bread resulted in significant differences for $p< 0.05$ in the preference of the appearance $\chi^2(3) = 8.371$, $p = 0.039$ while no impact was observed for the aroma preference $\chi^2(3) = 1.286$ $p= 0.733$. Furthermore, based on the LSRD, the control bread samples B1 and the samples containing 10 g of tryptic hydrolysate B4 were significantly preferred over the samples B3; in contrast, no significant differences were observed between the samples B2, B3 and B4 (**Table 27**). These findings suggested that there was not a reciprocal trend towards the preference of the panelists and the increasing hydrolysate concentration in the case of trypsin. Moreover, for the general appearance, the panelists commented that it was very hard to spot visual differences between the samples.

The use of the whey protein hydrolysate obtained from the digestion with proteinase from *Aspergillus melleus* produced a clear impact for bread samples. The results of the test depicted in **Table 27** indicate that there were significant differences ($p< 0.05$) among the samples preference, with $\chi^2(3) = 9.971$, $p = 0.019$, and $\chi^2(3) = 23.571$, $p< 0.001$ for the appearance and the aroma, respectively. The appearance of the control bread samples B1 was significantly preferred over the samples B6 and B7, respectively. The aroma of the control bread samples B1 and the samples containing 2,5g of whey protein hydrolysate from *A. melleus* (B5) were significantly preferred over the aroma of samples B6 and B7 (**Table 27**). These differences in preference towards the lowest concentrations and the controls were attributed to a dark, burnt appearance and an irregular crumb (**Figure 17**) of the bread samples containing high quantities of the hydrolysate from *A. melleus*, which was visible even under red light. Furthermore, the panelists reported a characteristic smell in these samples making them undesirable. Some panelists commented that the odor of samples B7 was quite intense and described it as a grilled cheese-like aroma. Therefore, it was clear that the trypsin hydrolysate had a less pronounced impact on the aroma of bread compared to the hydrolysate obtained with proteinase from *Aspergillus melleus*.

Table 27. Summary of the sensory analysis of bread samples

Bread samples containing tryptic hydrolysate								
	Appearance				Aroma			
Sample	B1	B2	B3	B4	B1	B2	B3	B4
Rank Sum	63 a	50 ab	40 b	57 a	51 a	49 a	52 a	58 a
Mode	4	1	2	3	1	2	2	4
Min value	1	1	1	1	1	1	1	1
Max value	4	4	4	4	4	4	4	4
Bread samples containing hydrolysate from *A. melleus* proteinase								
	Appearance				Aroma			
Concentration %	B1	B5	B6	B7	B1	B5	B6	B7
Rank Sum	65 a	58 ab	43 b	44 b	71 a	59 ac	48 bc	32 b
Mode	4	3	2	1	4	3	2	1
Min value	1	1	1	1	1	1	1	1
Max value	4	4	4	4	4	4	4	4

Values in the same row followed by different letters are significantly different in ranked preference. Highest rank sum score corresponds to the highest preference. The minimum difference in rank sums for two samples to be significantly different was calculated, LSRD = 16.4 for α= 0.05

The use of tryptic hydrolysate, in cookie samples resulted in significant differences ($p< 0.05$) among the preference, $\chi^2(3) = 28.371$, $p < 0.001$ and $\chi^2(3) = 21.286$, $p = 0.001$ for the appearance and the aroma respectively (**Table 28**). Identical preference patterns were observed both for the appearance and the aroma of cookies, where the control samples C1 and the samples C2 and C3 containing 2,35 g and 4.7 g of tryptic hydrolysate respectively, were significantly preferred over the samples C4 containing 9.4 g. The obtained results revealed that the sensory impact of the tryptic hydrolysate was very subtle. Panelists were only able to distinguish differences in cookies C4 when a significant amount of the hydrolysate was used. Additionally, at concentrations of 2.35 g, the panel characterized its aroma as slightly malty and baked.

The use of the hydrolysate from *A. melleus* proteinase in cookie samples, resulted in significant differences ($p< 0.05$) among the preference, $\chi^2(3) = 27.743$, $p < 0.001$ and $\chi^2(3) = 27.514$, $p < 0.001$ for the appearance and the aroma respectively. The appearance of the control samples C1 and the samples C5 and C6 were significantly preferred over the samples C7 containing 9.4 g of this hydrolysate. The aroma of the control samples C1 was significantly preferred over the samples C6 and C7, while no significant differences were observed between the samples C1 and C5. In addition, cookie samples C5 and C6 were significantly preferred over the cookies C7 (**Table 28**). A number of comments from the sensory panel reported that the cookies C7, containing 9.4 g of the hydrolysate

from *A. melleus* proteinase had a prominent burnt off odor. Interestingly, the sensory results from the aroma preference indicate that the use of the hydrolysate from *A. melleus* proteinase had a substantial impact on the aroma of the cookies in comparison with the control samples. However, its use in high concentrations gave rise to a noticeable undesirable effect.

Table 28. Summary of the sensory analysis of cookie samples

Cookie samples containing tryptic hydrolysate								
	Appearance				Aroma			
Sample	C1	C2	C3	C4	C1	C2	C3	C4
Rank Sum	64 a	67 a	52 a	27 b	68 a	59 a	52 a	31 b
Mode	4	4	3	1	4	4	2	1
Min value	1	1	1	1	2	1	1	1
Max value	4	4	3	3	4	4	4	4
Cookie samples containing hydrolysate from *A. melleus* proteinase								
	Appearance				Aroma			
Sample	C1	C5	C6	C7	C1	C5	C6	C7
Rank Sum	69 a	59 a	55 a	27 b	70 a	60 ab	52 b	28 c
Mode	4	2	3	1	4	3	2	1
Min value	1	2	1	1	1	1	2	1
Max value	4	4	4	4	4	4	4	4

Values in the same row followed by different letters are significantly different in ranked preference. Highest rank sum score corresponds to the highest preference The minimum difference in rank sums for two samples to be significantly different was calculated, LSRD = 16.4 for α= 0.05

Considering the overall quantities of whey protein hydrolysates used in the formulation of both food products, it is remarkable that a small addition of whey protein hydrolysate could have such impact on the generation of 2,5-dimethylpyrazine among other volatiles for both tested products. The results previously presented, indicate that for both food products, the use of hydrolyzed whey protein was effective in generating flavor compounds that were not present in the respective control samples (**Table 24**).

The use of the hydrolysate from *A. melleus* proteinase, produced remarkable differences depending on its concentration. As it can be observed in **Table 25**, the production of 2,5-dimethylpyrazine had a considerable upward tendency for both food products. At the same time, as observed in **Table 28** the sensory panel described the appearance of the samples C4 and C7 as burnt compared to the control

samples, which is obviously due to the increased production of melanoidins due to the Maillard reaction. However, this observation was not so clear for bread samples (**Table 27**), probably because the majority of the melanoidins are formed in the bread crust, while no clear differences could be observed in the crumb. Further, the addition of the proteolytic hydrolysate generated flavor compounds that the sensory panel considered undesirable. These aromas were described as "grilled cheese" and "pungent" flavors. This production of undesirable aromas in these food products can be explained as a direct consequence of the addition of the whey protein hydrolysate from *A. melleus*. Firstly, due to Maillard reactions, the high concentration of free amino acids in the hydrolysate could generate a variety of Strecker aldehydes like 2-methylpropanal and 3-methylbutanal, both aldehydes being generated through the Strecker degradation of valine and leucine (**Scheme 5, Scheme 6**), which are present in significant quantities in this hydrolysate.

As can be seen in **Table 24**, butanal, 2-methylbutanal and 3-methylbutanal were detected preliminary in a full scan of the cookie samples containing whey protein hydrolysate from *A. melleus*. Although 2-methylpropanal was not detected, most likely due to overlapping, it is probable that it was present as well due to the considerable quantities of valine in this hydrolysate. Methional was also detected in the cookie samples containing the whey protein hydrolysate from *A. melleus*. This compound is generated from the degradation of methionine, also present in the free amino acid composition of the hydrolysate. Methional has been reported to have a strong cabbage, potato and sulfur aroma,[196] and an extremely low odor threshold value, capable to contribute significantly to the overall aroma at the part per billion level or lower.[59,68] Other volatiles that were detected in the preliminary scan could contribute as well to the cheese-like odors described by the panelist (**Table 24**). On the other hand, it has been reviewed recently that the perception of mixtures of odorants, even if each is correctly identified alone, is not just a simple sum of the percepts of the individual components. Furthermore, for mixtures containing more than four components, the odorants were found to lose their individuality and produce a new odor. Despite of the fact that it is difficult to identify individual odorants in a mixture, humans can easily discriminate mixtures from each other.[82] Nevertheless, it must be noticed that the primary objective this investigation was to evaluate the formation of 2,5-dimethylpyrazine as consequence of the use of protein hydrolysates in food products.

Regarding the use of whey protein hydrolysate obtained upon a tryptic digestion, the production of Maillard volatiles and melanoidins was less pronounced. As deducted from **Table 27** and **Table 28**, the sensory panel had more difficulties to differentiate samples containing different concentrations of tryptic hydrolysate. At the same time, the results depicted in **Table 25** show that the production of 2,5-dimethylpyrazine presented a less pronounced increasing trend. However, the main difference between the uses of these two hydrolysates is the fact that the sensory panel did not describe the

samples containing tryptic hydrolyzed whey as unpleasant. Thus, the expected production of pyrazines was not accompanied by the same generation of amino acid specific volatiles which were present in the samples containing proteolytic hydrolysate (**Table 24**). As observed in **Table 17**, the tryptic hydrolysate contained much lower quantities of free amino acids compared to the hydrolysate from *A. melleus* proteinase. Therefore, the production of Maillard volatiles was mainly due to the peptides present in the hydrolysate. Similar results were obtained in model systems containing hydrolyzed whey protein and glucose (**Chapter 2, Chapter 4**). Since peptides cannot follow the Strecker degradation mechanism, the formation of pyrazines from peptidic sources did not generate Strecker aldehydes, which are known to have strong flavor properties. Hence, this explains why the sensory panel only described roasted, baked or malty flavors, which are associated with pyrazines in samples containing tryptic hydrolyzed whey.

5.4 CONCLUSIONS

As is generally known, the production of different odors in food can determine their potential acceptability. Recently, Pacynsky et al. expressed that gluten-free bread does not contain some bread key-odorants like 2-acetyl-1-pyrroline and pyrazines, and therefore it appears as less attractive for potential consumers.[197] Furthermore, the authors reported that the use of different amino acids and sugars improved the aroma of gluten-free bread.[197] However, the use of peptides was not considered. As from this investigation, the use of whey protein hydrolysates was effective to generate 2,5-dimethylpyrazine in real foods. The GC-MS and sensory analysis showed that the whey protein hydrolysate from *Aspergillus melleus* had a greater impact in the flavor enhancement of the tested food products, while tryptic hydrolyzed whey had a milder effect. However, in both cookies and bread samples, the production of 2,5-dimethylpyrazine in samples containing the hydrolysate from *A. melleus* proteinase, was followed by a production of several other volatiles that were perceived as undesirable by the sensory panel. Moreover, when this particular hydrolysate was used, the appearance of both tested products was undesirable as well.

For cookie samples containing tryptic hydrolysate, the sensory panel mainly reported roasted and malty flavors which are typically associated with the generation of pyrazines like 2,5-dimethylpyrazines. Moreover, the results showed that the addition of 2.35 g of tryptic hydrolysate (1% of the food composition) resulted in an acceptable product for a considerable number of the panelists. Nevertheless, the use of tryptic hydrolysate in higher amounts resulted unpleasant. The impact of the tryptic hydrolysate in the aroma and appearance of bread could not be observed as the panel was not able to differentiate the samples.

It can be concluded that the peptides contained in the tryptic hydrolysate were appropriate flavor precursors, which generated 2,5-dimethylpyrazine without an excessive formation of undesirable volatiles. Therefore, whey protein hydrolysates, or other hydrolysates can be intentionally added to a food formulation to increase its roasted or malty character during different thermal processes common in the manufacturing of baked food products.

CHAPTER 6

General discussion and future perspectives

6.1 GENERAL CONCLUSIONS

The Maillard reaction has proven to be an incredibly complex reaction capable of generating hundreds of flavor compounds. Alkylpyrazines, which are Maillard volatiles, are important flavor compounds due to their behavior as odorants in several food products. The Maillard reaction was found to be influenced by several factors such as time, temperature, concentration of the reactants, pH and a_w, thereby affecting the overall production of different compounds.

This PhD dissertation has focused exclusively on pyrazines, trying to understand and elucidate the factors, mechanisms and synergistic activity thereof that influence their formation. The production of pyrazines was studied in Maillard model systems containing free amino acids, six different whey protein hydrolysates, native whey protein, glucose and two dicarbonyl compounds (methylglyoxal and pentane-2,3-dione). Factors such as thermal condition, a_w, concentration and type of the reagents, were evaluated for all the studied model systems. The results were further applied in real food matrices.

Several conclusions can be obtained from the overall work of this PhD dissertation. Nevertheless, they will be divided by considering two aspects: conclusions intended for the scientific community and conclusions that might be relevant for the industry.

Conclusions for the scientific community: aspects related to the construction of model systems

Several working conditions were evaluated to determine the optimal model, aqueous or dry models, temperature, ratio and type of reagents, and a_w.

Initially, two model systems were made with the intention of imitating two well-known industrial processes, commercial sterilization and roasting. As it is known, in such processes the transmission of the heat has to be uniform. The sterilization-based models were relatively easy to develop because the reactants were dissolved in phosphate buffer making the mixture homogeneous. Moreover, an effective transmission of heat could be achieved using an oil bath, while the liquid samples were constantly mixed with a magnetic stirrer. However, the models that were designed to imitate roasting conditions involved dry heating over the dry mixtures of reagents and this showed to be problematic since the homogeneity of the samples was not constant, therefore, the results were not reproducible. For such reason, in the dry roasting model systems, the samples were dissolved and put in SPME vials containing sea sand. Further, the samples were freeze-dried and capped and this

improved the reproducibility of the results considerably. For such reason, it is important to consider that in order to study model systems, the first aspect that has to be taken into account it is its correct design, this, in order to have reproducible results.

After the reproducibility issue of the Maillard model systems was solved, both heating conditions were evaluated among other working conditions as a_w. It was shown that the highest production of pyrazines was found in model systems at low a_w (0.33) and high temperatures (> 120 ^oC).

Factors such as time, temperature, a_w and type of reagents were investigated extensively. However, another factor that plays an important role in the production of pyrazines is the ratio between reagents. The results presented in this dissertation showed that the optimal ratio between free amino acids and glucose is different to the optimal ratio of tryptic hydrolyzed whey and glucose (**Chapter 2**). Therefore, it was not surprising that this phenomenon was observed again in **Chapter 4** for a particular hydrolysate that contained a considerable amount of free amino acids, obtained upon enzymatic digestion with proteinase from *Aspergillus melleus*.

In general, for experiments with hydrolysates, it was found that when the ratio between hydrolyzed whey protein and glucose was low (ratio 1:0.08 and 1:0.16 w:w) the generation of pyrazines was significantly hindered. This effect can likely be explained by the low amount of dicarbonyl compounds generated from glucose degradation, which had an impact on the formation of pyrazines. The formation of pyrazines was higher for ratios up to 1:0.5 and 1:0.67 (w:w). Further, at higher protein/glucose ratios, a decrease in the formation of pyrazines was again observed. This may be due to the excessive formation of different carboxylic acids such as acetic acid from glucose,[150] which might decrease the pH of the model systems. It is generally known that the pH has an impact on the generation of pyrazines,[8] hindering their formation at low pH due to the protonation of the amino groups of the amino acids and peptides. Additionally, the low pH directly affects the generation of pyrazines while favoring the 1,2-enolization mechanism of the Amadori or Heyns products, over the 2,3-enolization pathway in which the precursors for pyrazine generation are formed (**Section 1.4.2**)

The reasons for which it is important to find the optimal ratio between the reagents in a Maillard model system, is the fact that an incorrect balance might hinder the production of pyrazines. Therefore, this could lead to the issue of an incorrect design of a Maillard model system.

Conclusions for the scientific community: the role of peptides in Maillard model systems

Upon determination of the optimal working conditions, another experiment was made to assess the role and contribution of peptides and amino acids present in a protein hydrolysate to generate

pyrazines. Model systems containing hydrolyzed whey were compared to model systems containing native whey, and native whey plus the addition of free amino acids in quantities corresponding to those generated upon tryptic digestion of the whey protein. The results of this experiment demonstrated that the peptides present in the hydrolysate were the main precursors for the formation of pyrazines, while the role of the amino acids was minor (**Chapter 2**). These results were further confirmed with five other hydrolysates, for which the same experimental conditions were used. In all cases, the peptides present in the hydrolysates were the main contributors for the pyrazine formation. However, for one particular hydrolysate obtained upon enzymatic hydrolysis with proteinase from *Aspergillus melleus*, in this case the contribution of free amino acids was almost the same as the contribution of peptides for the generation of pyrazines. Nevertheless, this hydrolysate contained high quantities of free amino acids as compared to the other hydrolysates that were used in the experiments.

Therefore, an important conclusion to take is the fact that amino acids are important pyrazine precursors and their contribution cannot be neglected. However, we see in the practice that food does not contain high quantities of free amino acids. Moreover, the experiments presented in **Chapters 2** and **4** showed that peptides were adequate pyrazines precursors as well.

It was clear that hydrolysates are capable to generate pyrazines, mainly due to the presence of peptides. Therefore, the tryptic hydrolysate was divided in six fractions of different molecular weight using preparative gel permeation chromatography and each fraction was characterized by their capacity to generate pyrazines in Maillard model systems. It was found that all the different fractions were able to generate pyrazines, producing more in low molecular weight fraction, most likely due to an increased amount of *N*-terminal amino acids to form aminoketones. Heavier fractions (Frac 1 and Frac 2) generated lower and statistically similar amounts of pyrazines between them. This suggests that high molecular weight peptides might not be the most suitable pyrazine precursors, as they were found to have low reactivity. Moreover, a satisfactory explanation for this low reactivity could not be given. It is hypothesized that it could be linked to factors such as steric hindrance or less availability of the N-terminal amino acids due to peptide aggregation or formation of secondary structures. Nevertheless, further research is necessary in this field.

Another important factor that needs to be considered for model systems containing peptides is their sequence, more exactly their *N*-terminus amino acids. Peptides that contain proline or hydroxyproline in the N-terminus are not able to follow the mechanism proposed by Van Lancker et al.[120] due to the absence of a primary amino group. Moreover, the same author reported that peptides that contain valine, leucine or isoleucine were less effective in the generation of pyrazines, most likely due to steric hindrance.[121]

Conclusions for the scientific community: the chemistry of pyrazine formation in complex model systems

Different pyrazines were generated in all the investigated model systems. Therefore, the mechanisms involved in the generation of there pyrazines were nvestigated as well. Significant quantities of the following pyrazines were produced in all model systems containing hydrolyzed whey protein (or its fractions) and glucose: 2-methylpyrazine, 2,5(6)-dimethylpyrazine, 2-ethyl-3(6)-methylpyrazine, 3-ethyl-2,5-dimethylpyrazine and 2-ethyl-3,5-dimethylpyrazine. At the same time, some pyrazines such as 2,5-dimethyl-3-(3-methylbutyl)pyrazine and 2,5-dimethyl-3-(2-methylbutyl)pyrazine which are known to be amino acid specific, were formed mostly in model systems in which the amount of free amino acids (particularly leucine and isoleucine) present was high.

In all the studied model systems, 2,5(6)-dimethylpyrazine and 3-ethyl-2,5-dimethylpyrazine were the most abundant pyrazines. The abundance of 2,5(6)-dimethylpyrazine can be explained as a direct consequence of the intermediate stage of the Maillard reaction. When glucose, after the Amadori rearrangement undergoes 2,3-enolysation, it generates methylglyoxal as a cleavage product of the rearranged sugar in high quantities. Further, whenever methylglyoxal reacts with free amino acids or the *N*-terminal amino acid of a peptide or protein, it forms 1-aminopropan-2-one. Thus, the condensation of two molecules of 1-aminopropan-2-one generates 2,5(6)-dimethylpyrazine. Therefore, a high abundance of methylglyoxal leads to the formation of 2,5(6)-dimethylpyrazine in high amounts. For such reason, it was not surprising to find 2,5(6)-dimethylpyrazine abundantly in all the model systems which were considered in this dissertation, including the experiments that were made using real food matrices.

The formation mechanisms leading to the formation of 3-ethyl-2,5-dimethylpyrazine and several other pyrazines were investigated in model systems containing whey protein hydrolysates and glucose (**Section 4.3.3**), and in two simplified models containing whey protein hydrolysates and dicarbonyl compounds (**Section 4.3.4**). The results obtained from these experiments demonstrated that several mechanisms lead to the formation of pyrazines, especially because α-amino ketones can be generated due to Strecker degradation of amino acids, reactions with peptides, and decarbonylation and decarboxylation of amino acids that contain an α-hydroxy group. Additionally, the various mechanisms that are responsible for specific alkyl substitutions in the pyrazine ring were proven, including self-condensation of amino ketones, thermal degradation of dicarbonyl compounds, aldol-type reactions of Strecker aldehydes, dicarbonyl elongation reactions and several

others. Moreover, the catalytic effect of lysine was observed for two model systems, suggesting that some amino acids might influence the pyrazine formation from other precursors such as peptides. Moreover, this demonstrates the complexity of volatile generation in food-related model systems, and therefore, in food itself.

Conclusions intended for the industry

Whey proteins are an important side product of the cheese and dairy industry. They are used in a variety of foods, including baby food, biscuits, ice cream, sport supplements and many other products for different reasons, such as nutritional or technological uses. The results presented in this PhD thesis suggest that whey proteins could be used as flavor promoters as well.

Native and hydrolyzed whey protein isolate was used to develop Maillard model systems to study the generation of pyrazines. The relatively low cost and high protein content of the whey protein isolates proved to be suitable to perform experiments under a considerable variety of conditions. In spite of the fact that whey protein can be obtained from the dairy industry, the Maillard model systems studied in **Chapter 2**, **Chapter 3** and **Chapter 4** are not likely to be applicable to this industry. In dairy products, the formation of pyrazines in generally undesirable and their formation indicate inadequate processing.

It is considered that the mixtures of protein or peptides and glucose can be intentionally added to products in which it is desirable to promote the formation of pyrazines. Such products could be meat substitutes to improve the characteristic roasting flavor of meat, or baked snack products in which smoky-roasted flavors are expected as part of its characteristic flavor as for example barbecue sauce and smoky flavored chips or peanuts.

Different peptidases were used to hydrolyze whey protein, as a result, hydrolysates containing different peptides and amino acids were generated. Moreover, this allowed performing different Maillard model systems. For the set of reactions leading to the formation of pyrazines, peptides were the top contributors. An important aspect derived from this conclusion, is that since in real food, specially fermented or partially hydrolyzed, free amino acids are not the only contributors to the formation of pyrazines, peptides were proven to contribute to their formation as well.

Several pyrazines were detected in previously evaluated model systems, 2,5-dimethylpyrazine being the most abundant. Therefore, it was likely that this pyrazine could be detected in real food experiment as well. Many factors can influence pyrazine generation as a result of the Maillard reaction in model systems or food products; and, as the matrix where the reaction takes place becomes more complex, more factors can influence it.

As it was described in the introduction of **Chapter 5**, the transfer from a model system to a food model results often problematic. One aspect that was not considered in the model systems but that possesses a remarkable importance in food products is the presence of lipids. As it was reported by Zamora and Hidalgo, lipid oxidation products can interact with Maillard products as well.[182] Moreover, two dicarbonyl compounds, glyoxal and methylglyoxal, which are generated upon the dehydration and cleavage pathway in the intermediate stage of the Maillard reaction (**Section 1.4.2.1**), can be formed as lipid oxidation products as well.[182] Lipid oxidation products are able to generate important flavor compounds and influence the Maillard reaction. Therefore, the presence of lipids is critical to consider for food model experiments. Nevertheless, in the particular case of pyrazines, there is little evidence that link the generation of these volatiles as result of the influence of lipids. Moreover, the use of protein hydrolysates to generate pyrazines in food models was successful.

The formation of pyrazines as consequence of peptide addition was evaluated in two real food models (**Chapter 5**). Whey protein hydrolysates obtained from digestion with trypsin and proteinase from *Aspergillus melllus* was added to the formulation of bread and cookies, with the intention of promoting the generation of pyrazines. Both hydrolysates were able to generate pyrazines, 2,5-dimethylpyrazine being the most abundant. Therefore, this pyrazine was selected to evaluate the impact of protein hydrolysate addition on the generation of pyrazines in real food models. The experimental conditions used in the food experiments were milder than the conditions used for the studied model systems. This demonstrated that hydrolysates were appropriated precursors to study the generation of pyrazines as products of the Maillard reaction in real food systems.
The resulting food samples were further evaluated by a sensory panel to determine their acceptability. Considering the overall quantities of whey protein hydrolysates used in the formulation of both food products, it was remarkable that a small addition of whey protein hydrolysate could have a significant impact on the generation of 2,5-dimethylpyrazine for both tested products. Although other volatiles were generated as well, the sensory panel was able to clearly differentiate the samples it preferred except for bread samples containing tryptic hydrolyzed whey.

Regarding the tested food models of **Chapter 5**, it can be concluded that the peptides contained in the hydrolysate were appropriate flavor precursors to generate 2,5-dimethylpyrazine. It is most likely that other pyrazines can be formed as well. Therefore, whey protein hydrolysates could be intentionally added to a food formulation to increase its roasted or malty character.

6.2 PROSPECTIVE WORK

Over the course of the present work, the mechanism of amino ketone formation suggested by Van Lancker et al.[120] was of enormous importance and provided a better understanding of how pyrazines get generated in complex systems. However, this mechanism was proposed but not validated. For this reason, this pathway which leads to the formation of pyrazines from peptides need further study. A possible experimental approach could be the use of peptides in which the *N*-terminal amino acid is labeled with ^{15}N. Further, the use of ^{15}N-labeled amino acids could be an advantage in the effort to elucidate these complex mechanisms of the pyrazine generation: one possibility could be to use a ^{15}N-labeled amino acid as the middle amino acid in a tri- or pentapeptide. This would allow to determine if some experimental conditions promote peptide hydrolysis and further generation of pyrazines.

As was observed in the last experiment, peptides can be used as pyrazine precursors on baked products. A work perspective in this aspect could be to continue experiments with other food products in which pyrazines are expected, or better even, desired. Meat substitutes based in vegetal protein sources could be an appropriate model to be investigated, particularly since it might help to imitate the flavor characteristics of animal meat products. Moreover, a more complete sensory evaluation of appearance, aroma and taste could be studied in several foods if the peptide hydrolysates are obtained with food grade enzymes. Other prospective work could be to work not only with different food products but also with different hydrolysates and proportions of mixtures. This would further allow for the discovery of the optimal concentrations that lead to pleasant aromas without affecting other properties. During the elaboration process of bread, it could be observed that the use of non-hydrolyzed whey protein produced a strong crumb, most likely due to an increase in the viscosity of the dough. This effect was not observed in the samples in which the whey protein hydrolysates were used. As a result, it would be interesting to evaluate the texture and rheology of the products containing protein hydrolysates.

Considering some aspects related to human nutrition, whey proteins are one of the most common allergens reported in food products. Therefore, studies concerning the allergenicity of the hydrolysates and their Maillard products could be tested as well. Additionally, it is known that human digestive peptidases are quite specific in their cleavage mechanisms. Therefore, it would be interesting to investigate their action on Maillard products of proteins or peptides. Analysis of in vitro digestibility in vitro and other properties like membrane transport could be suitable these purposes.

CHAPTER 7

Appendix

A GUIDE TO THE TWENTY COMMON AMINO ACIDS

AMINO ACIDS ARE THE BUILDING BLOCKS OF PROTEINS IN LIVING ORGANISMS. THERE ARE OVER 500 AMINO ACIDS FOUND IN NATURE - HOWEVER, THE HUMAN GENETIC CODE ONLY DIRECTLY ENCODES 20. 'ESSENTIAL' AMINO ACIDS MUST BE OBTAINED FROM THE DIET, WHILST NON-ESSENTIAL AMINO ACIDS CAN BE SYNTHESISED IN THE BODY.

Chart Key: ALIPHATIC · AROMATIC · ACIDIC · BASIC · HYDROXYLIC · SULFUR-CONTAINING · AMIDIC · NON-ESSENTIAL · ESSENTIAL

Chemical Structure — single letter code

NAME (A) — *three letter code* — DNA codons

ALANINE (A) *Ala* GCT, GCC, GCA, GCG	GLYCINE (G) *Gly* GGT, GGC, GGA, GGG	ISOLEUCINE (I) *Ile* ATT, ATC, ATA	LEUCINE (L) *Leu* CTT, CTC, CTA, CTG, TTA, TTG	PROLINE (P) *Pro* CCT, CCC, CCA, CCG	VALINE (V) *Val* GTT, GTC, GTA, GTG	
PHENYLALANINE (F) *Phe* TTT, TTC	TRYPTOPHAN (W) *Trp* TGG	TYROSINE (Y) *Tyr* TAT, TAC	ASPARTIC ACID (D) *Asp* GAT, GAC	GLUTAMIC ACID (E) *Glu* GAA, GAG	ARGININE (R) *Arg* CGT, CGC, CGA, CGG, AGA, AGG	HISTIDINE (H) *His* CAT, CAC
LYSINE (K) *Lys* AAA, AAG	SERINE (S) *Ser* TCT, TCC, TCA, TCG, AGT, AGC	THREONINE (T) *Thr* ACT, ACC, ACA, ACG	CYSTEINE (C) *Cys* TGT, TGC	METHIONINE (M) *Met* ATG	ASPARAGINE (N) *Asn* AAT, AAC	GLUTAMINE (Q) *Gln* CAA, CAG

Note: This chart only shows those amino acids for which the human genetic code directly codes for. Selenocysteine is often referred to as the 21st amino acid, but is encoded in a special manner. In some cases, distinguishing between asparagine/aspartic acid and glutamine/glutamic acid is difficult. In these cases, the codes asx (B) and glx (Z) are respectively used.

Appendix 2: Whey protein isolate composition

ArlaFoodsIngredients
Product sheet 0060

Date 09-01-15

Description

Lacprodan® DI-9224 is a functional whey protein isolate for protein fortification of clinical-, sports- and nutrition products. It has a high heat stability and is clear in solution, which makes it especially suited for pasteurized or UHT liquid products.

Properties

- Highly soluble over a wide pH-range
- Designed for solutions with a neutral pH
- Gives clear solutions with low viscosity
- Neutral taste
- Low fat and carbohydrate content

Application

1. Clinical nutrition
   - Sip feeding
2. Sports products
3. Low lactose infant formula

Chemical specifications

Protein (Nx6.38) as is	min	88 %
Protein (Nx6.38) d.m.	min	92 %
Lactose	Max	0.2 %
Fat	max/level	0.2 %
Ash	max/level	4.5 %
Moisture	Max	6.0 %

Minerals

Sodium	Na	level	0.5 %
Phosphorus	P	level	0.2 %
Chloride	Cl	level	0.05 %
Potassium	K	level	1.3 %
Calcium	Ca	level	0.1 %

Nutritional data

Calculated values for nutrition labeling per 100 g powder

Energy	1459 kJ/347 kcal

Physical specifications

pH (10 % solution)		6.5-7.0
Scorched particles		disc A
Bulk density (625)	level	0,4 g/cm^3
Solubility index	max	0.1 ml
Colour of powder		white to cream
Colour of 10% solution		clear
Flavour/odour		bland

Microbiological specifications

Total plate count	max	1,000 CFU/g
Enterobacteriaceae	absent in	10 CFU/g
Bacillus	max.	100 CFU/g
Staphylococcus aureus coagulase+	absent in	in 1g
Yeast/Mould	max	10 CFU/g
Salmonella	absent in	125 g

Amino acids (AA)	Typical amino acid composition g. AA/100 g. protein	
Alanine		5.6
Arginine		2.2
Aspartic acid (asparagine)		11.5
Cysteine (Cystine)		2.5
Glutamic acid (glutamine)		18.6
Glycine		1.7
Histidine	*	1.8
Isoleucine	*	7.1
Leucine	*	11.5
Lysine	*	10.3
Methionine	*	2.5
Phenylalanine	*	3.2
Proline		6.9
Serine		5.2
Threonine	*	7.7
Tryptophane	*	1.8
Tyrosine	*	3.1
Valine	*	6.3
Total BCAA/TAA		22.7
* Essential Aminoacids		

Packaging
Paper bags with a polyethylene inner liner containing 15 kg net.

Storage
Store in closed bags under cool and dry conditions to prevent deterioration due to humidity and high temperatures.

Shelf Life
Minimum 24 months if kept under the prescribed storage conditions.

Legal references
Lacprodan® DI-9224. The product is manufactured, packaged and labelled according to the relevant EU-regulations for food and food ingredients, and/or FAO/ WHO Codex Alimentarius, when relevant. This includes that the milk/milk constituents used as raw material origins from healthy cows. The milk used in the production is included in monitoring programmes for undesirable substances, as required by regulations or HACCP-based risk assessment. The production plants are approved by the competent authorities and included in the EU-register of approved food establishments.

For products manufactured outside EU the products comply with relevant regulations in the country where the product is produced.

Appendix 3: Predictive enzymatic hydrolysis of α-lactalbumin and β-lactoglobulin using different enzymes.

Enzyme: Trypsin

Protein: Alpha-lactalbumin (Lactose synthase B protein) (Allergen Bos d 4)

Maximum number of missed cleavages (MC): 2

All cysteines are in reduced form. Methionine have been oxidized to form methionine sulfoxide (MSO).

Using monoisotopic masses of the occurring amino acid residues and giving peptide masses as $[M+H]^+$.

Mass	Position	#MC	Artif.modification(s)	Peptide sequence
7055.1716	36-98	2		GYGGVSLPEWVCTTFHTSGY DTQAIVQNNDSTEYGLFQIN NKIWCKDDQNPHSSNICNIS CDK
5540.6201	33-81	2		DLKGYGGVSLPEWVCTTFHT SGYDTQAIVQNNDSTEYGLF QINNKIWCK
5380.5742	30-77	2		ELKDLKGYGGVSLPEWVCTT FHTSGYDTQAIVQNNDSTEY GLFQINNK
5184.4142	36-81	1		GYGGVSLPEWVCTTFHTSGY DTQAIVQNNDSTEYGLFQIN NKIWCK
5010.3526	33-77	1		DLKGYGGVSLPEWVCTTFHT SGYDTQAIVQNNDSTEYGLF QINNK
4654.1467	36-77	0		GYGGVSLPEWVCTTFHTSGY DTQAIVQNNDSTEYGLFQIN NK
4043.7588	78-112	2	MSO: 109 4059.7537	IWCKDDQNPHSSNICNISCD KFLDDDLTDDIMCVK
3641.5862	82-113	2	MSO: 109 3657.5811	DDQNPHSSNICNISCDKFLD DDLTDDIMCVKK
3513.4913	82-112	1	MSO: 109 3529.4862	DDQNPHSSNICNISCDKFLD DDLTDDIMCVK
2847.4320	118-141	2		VGINYWLAHKALCSEKLDQW LCEK
2420.0428	78-98	1		IWCKDDQNPHSSNICNISCD K
2301.2423	114-133	2		ILDKVGINYWLAHKALCSEK
2240.1188	99-117	2	MSO: 109 2256.1137	FLDDDLTDDIMCVKKILDK
1889.7752	82-98	0		DDQNPHSSNICNISCDK
1831.9523	118-133	1		VGINYWLAHKALCSEK
1798.0373	113-127	2		KILDKVGINYWLAHK
1778.8815	128-142	2		ALCSEKLDQWLCEKL
1770.8288	99-113	1	MSO: 109 1786.8237	FLDDDLTDDIMCVKK
1669.9424	114-127	1		ILDKVGINYWLAHK
1665.7975	128-141	1		ALCSEKLDQWLCEK

1642.7338	99-112	0	MSO: 109 1658.7288	FLDDDLTDDIMCVK
1622.8570	20-32	2		EQLTKCEVFRELK
1379.7351	25-35	2		CEVFRELKDLK
1252.6354	20-29	1		EQLTKCEVFR
1200.6524	118-127	0		VGINYWLAHK
1147.5816	134-142	1		LDQWLCEKL
1034.4975	134-141	0		LDQWLCEK
1023.5291	25-32	1		CEVFRELK
745.4454	30-35	1		ELKDLK
653.3075	25-29	0		CEVFR
650.3178	128-133	0		ALCSEK
618.3457	20-24	0		EQLTK
616.4028	113-117	1		KILDK
549.2853	78-81	0		IWCK
488.3078	114-117	0		ILDK
389.2394	30-32	0		ELK
375.2238	33-35	0		DLK
147.1128	113-113	0		K
132.1019	142-142	0		L

Enzyme: Trypsin

Protein: Beta-lactoglobulin precursor (Beta-LG) (Allergen Bos d 5)

Maximum number of missed cleavages (MC): 2

All cysteines are in reduced form. Methionine have been oxidized to form methionine sulfoxide (MSO).

Using monoisotopic masses of the occurring amino acid residues and giving peptide masses as $[M+H]^+$.

Mass	Position	#MC	Artif.modification(s)	Peptide sequence
6047.0455	31-85	2	MSO: 40 6063.0405	VAGTWYSLAMAASDISLLDA QSAPLRVYVEELKPTPEGDL EILLQKWENGECAQK
5655.9869	25-76	2	MSO: 40 5671.9818	GLDIQKVAGTWYSLAMAASD ISLLDAQSAPLRVYVEELKP TPEGDLEILLQK
5001.6168	31-76	1	MSO: 40 5017.6117	VAGTWYSLAMAASDISLLDA QSAPLRVYVEELKPTPEGDL EILLQK
4276.2719	17-56	2	MSO: 23, 40 4308.2617	LIVTQTMKGLDIQKVAGTWY SLAMAASDISLLDAQSAPLR
4263.9592	118-154	2	MSO: 123 4279.9541	YLLFCMENSAEPEQSLACQC LVRTPEVDDEALEKFDK
4001.8639	117-151	2	MSO: 123 4017.8588	KYLLFCMENSAEPEQSLACQ CLVRTPEVDDEALEK
3873.7689	118-151	1	MSO: 123 3889.7638	YLLFCMENSAEPEQSLACQC LVRTPEVDDEALEK
3821.8620	108-140	2	MSO: 123 3837.8569	VLVLDTDYKKYLLFCMENSA EPEQSLACQCLVR
3486.7824	57-86	2		VYVEELKPTPEGDLEILLQK WENGECAQKK
3361.7460	25-56	1	MSO: 40 3377.7409	GLDIQKVAGTWYSLAMAASD ISLLDAQSAPLR
3358.6875	57-85	1		VYVEELKPTPEGDLEILLQK WENGECAQK
2789.4589	155-178	2	MSO: 161 2805.4538	ALKALPMHIRLSFNPTQLEE QCHI
2775.2972	117-140	1	MSO: 123 2791.2922	KYLLFCMENSAEPEQSLACQ CLVR
2707.3759	31-56	0	MSO: 40 2723.3708	VAGTWYSLAMAASDISLLDA QSAPLR
2647.2023	118-140	0	MSO: 123 2663.1972	YLLFCMENSAEPEQSLACQC LVR
2618.4439	94-116	2		IPAVFKIDALNENKVLVLDT DYK
2477.2428	158-178	1	MSO: 161 2493.2377	ALPMHIRLSFNPTQLEEQCH I
2313.2587	57-76	0		VYVEELKPTPEGDLEILLQK
2091.1332	100-117	2		IDALNENKVLVLDTDYKK
1963.0382	100-116	1		IDALNENKVLVLDTDYK
1947.9909	141-157	2		TPEVDDEALEKFDKALK
1801.0218	92-107	2		TKIPAVFKIDALNENK
1746.8843	77-91	2		WENGECAQKKIIAEK
1658.7843	165-178	0		LSFNPTQLEEQCHI
1635.7748	141-154	1		TPEVDDEALEKFDK
1587.9138	17-30	1	MSO: 23 1603.9087	LIVTQTMKGLDIQK
1571.8791	94-107	1		IPAVFKIDALNENK
1539.8828	152-164	2	MSO: 161 1555.8777	FDKALKALPMHIR

1457.9090	87-99	2		IIAEKTKIPAVFK
1245.5845	141-151	0		TPEVDDEALEK
1193.6776	108-117	1		VLVLDTDYKK
1192.5415	77-86	1		WENGECAQKK
1149.6925	155-164	1	MSO: 161 1165.6874	ALKALPMHIR
1065.5826	108-116	0		VLVLDTDYK
1064.4465	77-85	0		WENGECAQK
933.5437	17-24	0	MSO: 23 949.5387	LIVTQTMK
930.5982	86-93	2		KIIAEKTK
916.4734	100-107	0		IDALNENK
903.5662	92-99	1		TKIPAVFK
837.4763	158-164	0	MSO: 161 853.4712	ALPMHIR
802.5032	87-93	1		IIAEKTK
721.4243	152-157	1		FDKALK
701.4556	86-91	1		KIIAEK
674.4235	94-99	0		IPAVFK
673.3879	25-30	0		GLDIQK
573.3606	87-91	0		IIAEK
409.2081	152-154	0		FDK
331.2340	155-157	0		ALK
248.1605	92-93	0		TK
147.1128	86-86	0		K
147.1128	117-117	0		K

Enzyme: Thermolysin

Protein: Alpha-lactalbumin (Lactose synthase B protein) (Allergen Bos d 4)

Maximum number of missed cleavages (MC): 2

All cysteines are in reduced form. Methionine have been oxidized to form methionine sulfoxide (MSO).

Using monoisotopic masses of the occurring amino acid residues and giving peptide masses as $[M+H]^+$.

Mass	Position	#MC	Artif.modification(s)	Peptide sequence
2767.4487	22-45	2		LTKCEVFRELKDLKGYGGVS LPEW
2498.2747	28-49	2		FRELKDLKGYGGVSLPEWVC TT
2420.0428	78-98	2		IWCKDDQNPHSSNICNISCD K
2343.0605	74-93	2		INNKIWCKDDQNPHSSNICN
2313.1907	20-39	2		EQLTKCEVFRELKDLKGYGG
2288.0513	72-90	2		FQINNKIWCKDDQNPHSSN
2170.9750	40-58	2		VSLPEWVCTTFHTSGYDTQ
2094.1018	28-45	1		FRELKDLKGYGGVSLPEW
2056.0895	22-39	1		LTKCEVFRELKDLKGYGG
2012.9243	74-90	1		INNKIWCKDDQNPHSSN
1873.7956	78-93	1		IWCKDDQNPHSSNICN
1727.7680	94-108	2		ISCDKFLDDDLTDDI
1623.7604	100-113	2	MSO: 109 1639.7553	LDDDLTDDIMCVKK
1594.7603	129-141	2		LCSEKLDQWLCEK
1543.6594	78-90	0		IWCKDDQNPHSSN
1530.6529	46-59	2		VCTTFHTSGYDTQA
1514.6758	61-73	2		VQNNDSTEYGLFQ
1459.6158	46-58	1		VCTTFHTSGYDTQ
1415.5705	99-110	2	MSO: 109 1431.5654	FLDDDLTDDIMC
1382.7426	28-39	0		FRELKDLKGYGG
1352.6328	60-71	2		IVQNNDSTEYGL
1310.5859	59-70	2		AIVQNNDSTEYG
1268.5021	100-110	1	MSO: 109 1284.4970	LDDDLTDDIMC
1239.5640	50-60	2		FHTSGYDTQAI
1239.5488	60-70	1		IVQNNDSTEYG
1239.5488	61-71	1		VQNNDSTEYGL
1192.5667	128-137	2		ALCSEKLDQW
1181.5208	99-108	1		FLDDDLTDDI
1147.5816	134-142	2		LDQWLCEKL
1134.5499	40-49	1		VSLPEWVCTT
1126.4800	50-59	1		FHTSGYDTQA

1126.4647	61-70	0		VQNNDSTEYG
1121.5295	129-137	1		LCSEKLDQW
1107.5833	115-123	2		LDKVGINYW
1055.4428	50-58	0		FHTSGYDTQ
1044.5625	120-127	2		INYWLAHK
1042.4696	91-99	2		ICNISCDKF
1034.4975	134-141	1		LDQWLCEK
1034.4524	100-108	0		LDDDLTDDI
986.5087	125-133	2		AHKALCSEK
949.4659	20-27	1		EQLTKCEV
895.4012	91-98	1		ICNISCDK
876.4938	71-77	2		LFQINNK
864.4614	118-124	2		VGINYWL
843.5662	111-117	2		VKKILDK
763.4097	72-77	1		FQINNK
751.3773	118-123	1		VGINYW
730.3770	40-45	0		VSLPEW
721.4099	109-114	2	MSO: 109 737.4048	MCVKKI
712.3334	94-99	1		ISCDKF
708.3715	120-124	1		INYWL
692.3647	22-27	0		LTKCEV
650.3178	128-133	1		ALCSEK
644.3977	114-119	2		ILDKVG
608.3258	109-113	1	MSO: 109 624.3207	MCVKK
605.3327	138-142	1		LCEKL
595.2875	120-123	0		INYW
579.2807	129-133	0		LCSEK
565.2650	94-98	0		ISCDK
561.2667	134-137	0		LDQW
539.3300	124-128	2		LAHKA
531.3137	115-119	1		LDKVG
492.2486	138-141	0		LCEK
488.3078	114-117	1		ILDK
488.2827	74-77	0		INNK
487.3602	111-114	1		VKKI
468.2929	124-127	1		LAHK
426.2459	125-128	1		AHKA
423.1908	46-49	0		VCTT
407.2289	71-73	1		LFQ
375.2238	115-117	0		LDK
374.2762	111-113	0		VKK
355.2088	125-127	0		AHK
349.1540	91-93	0		ICN

294.1448	72-73	0		FQ
276.1190	20-21	0		EQ
253.0675	109-110	0	MSO: 109 269.0624	MC
203.1390	59-60	1		AI
175.1077	118-119	0		VG
166.0862	99-99	0		F
132.1019	60-60	0		I
132.1019	71-71	0		L
132.1019	114-114	0		I
132.1019	124-124	0		L
132.1019	142-142	0		L
90.0549	59-59	0		A
90.0549	128-128	0		A

Enzyme: Thermolysin

Protein: Beta-lactoglobulin precursor (Beta-LG) (Allergen Bos d 5)

Maximum number of missed cleavages (MC): 2

All cysteines are in reduced form. Methionine have been oxidized to form methionine sulfoxide (MSO).

Using monoisotopic masses of the occurring amino acid residues and giving peptide masses as $[M+H]^+$.

Mass	Position	#MC	Artif.modification(s)	Peptide sequence
2769.3651	59-82	2		VEELKPTPEGDLEILLQKWE NGEC
2100.1222	55-72	2		LRVYVEELKPTPEGDLEI
1944.0211	57-73	2		VYVEELKPTPEGDLEIL
1890.9443	139-154	2		VRTPEVDDEALEKFDK
1830.9371	57-72	1		VYVEELKPTPEGDLEI
1681.8894	59-73	1		VEELKPTPEGDLEIL
1674.8632	73-86	2		LLQKWENGECAQKK
1674.8632	74-87	2		LQKWENGECAQKKI
1648.7305	134-148	2		ACQCLVRTPEVDDEA
1613.8380	138-151	2		LVRTPEVDDEALEK
1568.8053	59-72	0		VEELKPTPEGDLEI
1561.7791	74-86	1		LQKWENGECAQKK
1545.7002	165-177	2		LSFNPTQLEEQCH
1500.7540	139-151	1		VRTPEVDDEALEK
1458.6682	167-178	2		FNPTQLEEQCHI
1380.7158	26-37	2		LDIQKVAGTWYS
1371.5191	121-132	2	MSO: 123 1387.5140	FCMENSAEPEQS
1361.7457	19-30	2	MSO: 23 1377.7406	VTQTMKGLDIQK
1345.5841	167-177	1		FNPTQLEEQCH
1287.6426	42-54	2		ASDISLLDAQSAP
1271.6881	111-120	2		LDTDYKKYLL
1257.6725	109-118	2		LVLDTDYKKY
1257.6725	110-119	2		VLDTDYKKYL
1243.6164	138-148	1		LVRTPEVDDEA
1234.5256	123-133	2	MSO: 123 1250.5205	MENSAEPEQSL
1232.6633	48-58	2		LDAQSAPLRVY
1219.5776	73-82	1		LLQKWENGEC
1191.6368	98-107	2		FKIDALNENK
1178.4816	127-137	2		AEPEQSLACQC
1158.6041	111-119	1		LDTDYKKYL
1144.5884	110-118	1		VLDTDYKKY

1130.5324	139-148	0		VRTPEVDDEA
1121.4415	123-132	1	MSO: 123 1137.4364	MENSAEPEQS
1106.4935	74-82	0		LQKWENGEC
1083.6156	47-56	2		LLDAQSAPLR
1075.5894	163-171	2		IRLSFNPTQ
1045.5200	111-118	0		LDTDYKKY
1031.5917	23-31	2	MSO: 23 1047.5867	MKGLDIQKV
1015.5418	100-108	2		IDALNENKV
1012.6401	87-95	2		IIAEKTKIP
970.5931	88-96	2		IAEKTKIPA
970.5316	48-56	1		LDAQSAPLR
956.5775	89-97	2		AEKTKIPAV
932.5233	23-30	1	MSO: 23 948.5182	MKGLDIQK
916.4734	100-107	1		IDALNENK
899.5560	88-95	1		IAEKTKIP
896.4512	31-38	2		VAGTWYSL
877.4811	18-25	2	MSO: 23 893.4761	IVTQTMKG
871.3978	172-178	1		LEEQCHI
868.4199	32-39	2		AGTWYSLA
857.5091	89-96	1		AEKTKIPA
850.4669	149-155	2		LEKFDKA
843.3375	120-126	2	MSO: 123 859.3324	LFCMENS
837.4763	158-164	2	MSO: 161 853.4712	ALPMHIR
829.4778	103-109	2		LNENKVL
814.4305	47-54	1		LLDAQSAP
809.4702	156-162	2	MSO: 161 825.4651	LKALPMH
806.4043	165-171	1		LSFNPTQ
797.3828	32-38	1		AGTWYSL
786.4719	89-95	0		AEKTKIP
783.3672	31-37	1		VAGTWYS
779.4297	149-154	1		LEKFDK
773.3675	127-133	1		AEPEQSL
764.3971	19-25	1	MSO: 23 780.3920	VTQTMKG
758.3137	172-177	0		LEEQCH
756.4185	161-166	2	MSO: 161 772.4134	MHIRLS
730.2535	121-126	1	MSO: 123 746.2484	FCMENS
721.4243	152-157	2		FDKALK
716.3937	103-108	1		LNENKV
715.4348	26-31	1		LDIQKV
701.3464	48-54	0		LDAQSAP
700.4716	83-88	2		AQKKII

694.3076	40-46	2	MSO: 40 710.3025	MAASDIS
692.3977	97-102	2		VFKIDA
684.2987	32-37	0		AGTWYS
676.3512	41-47	2		AASDISL
674.4083	17-22	2		LIVTQT
660.2835	127-132	0		AEPEQS
650.3000	133-138	2		LACQCL
617.3253	103-107	0		LNENK
616.3664	26-30	0		LDIQK
612.4079	155-160	2		ALKALP
606.2882	167-171	0		FNPTQ
605.3141	42-47	1		ASDISL
593.3293	98-102	1		FKIDA
587.3875	83-87	1		AQKKI
568.2912	158-162	1	MSO: 161 584.2861	ALPMH
563.2671	41-46	1		AASDIS
561.3242	18-22	1		IVTQT
556.3024	161-164	1	MSO: 161 572.2973	MHIR
550.3347	55-58	1		LRVY
541.3708	156-160	1		LKALP
537.2160	133-137	1		LACQC
537.2160	134-138	1		ACQCL
495.2635	119-122	2		LLFC
492.2300	42-46	0		ASDIS
488.3191	163-166	1		IRLS
480.2452	152-155	1		FDKA
480.1759	123-126	0	MSO: 123 496.1708	MENS
474.3034	83-86	0		AQKK
464.2867	96-99	2		AVFK
448.2402	19-22	0		VTQT
424.1319	134-137	0		ACQC
409.2081	152-154	0		FDK
393.2496	97-99	1		VFK
389.2394	149-151	0		LEK
382.1795	120-122	1		LFC
335.1747	23-25	0	MSO: 23 351.1696	MKG
334.1795	38-40	2	MSO: 40 350.1744	LAM
331.2340	155-157	1		ALK
330.2387	108-110	2		VLV
318.1659	100-102	0		IDA
300.1918	158-160	0		ALP
294.1812	98-99	0		FK

292.1325	39-41	2	MSO: 40 308.1274	AMA
288.2030	55-56	0		LR
288.2030	163-164	0		IR
287.1172	161-162	0	MSO: 161 303.1121	MH
281.1496	57-58	0		VY
269.0954	121-122	0		FC
260.1968	156-157	0		LK
245.1859	17-18	1		LI
245.1859	87-88	1		II
245.1859	119-120	1		LL
231.1703	108-109	1		VL
231.1703	109-110	1		LV
221.0954	39-40	1	MSO: 40 237.0903	AM
221.0954	40-41	1	MSO: 40 237.0903	MA
219.1339	165-166	0		LS
203.1390	38-39	1		LA
189.1233	96-97	1		AV
150.0583	40-40	0	MSO: 40 166.0532	M
132.1019	17-17	0		L
132.1019	18-18	0		I
132.1019	38-38	0		L
132.1019	47-47	0		L
132.1019	73-73	0		L
132.1019	87-87	0		I
132.1019	88-88	0		I
132.1019	109-109	0		L
132.1019	119-119	0		L
132.1019	120-120	0		L
132.1019	133-133	0		L
132.1019	138-138	0		L
132.1019	178-178	0		I
118.0862	31-31	0		V
118.0862	97-97	0		V
118.0862	108-108	0		V
118.0862	110-110	0		V
90.0549	39-39	0		A
90.0549	41-41	0		A
90.0549	96-96	0		A
90.0549	155-155	0		A

Enzyme: Pepsin (pH 1.3)

Protein: Alpha-lactalbumin (Lactose synthase B protein) (Allergen Bos d 4)

Maximum number of missed cleavages (MC): 2

All cysteines are in reduced form. Methionine have been oxidized to form methionine sulfoxide (MSO).

Using monoisotopic masses of the occurring amino acid residues and giving peptide masses as $[M+H]^+$.

Mass	Position	#MC	Artif.modification(s)	Peptide sequence
5605.5118	51-99	2		HTSGYDTQAIVQNNDSTEYG LFQINNKIWCKDDQNPHSSN ICNISCDKF
4037.8497	35-71	2		KGYGGVSLPEWVCTTFHTSG YDTQAIVQNNDSTEYGL
3735.6836	73-104	2		QINNKIWCKDDQNPHSSNIC NISCDKFLDDDL
3424.5871	72-100	2		FQINNKIWCKDDQNPHSSNI CNISCDKFL
3423.5110	43-72	2		PEWVCTTFHTSGYDTQAIVQ NNDSTEYGLF
3311.5030	72-99	1		FQINNKIWCKDDQNPHSSNI CNISCDKF
3277.5187	73-100	1		QINNKIWCKDDQNPHSSNIC NISCDKFL
3276.4426	43-71	1		PEWVCTTFHTSGYDTQAIVQ NNDSTEYGL
3164.4346	73-99	0		QINNKIWCKDDQNPHSSNIC NISCDKF
2887.5572	105-129	2	MSO: 109 2903.5521	TDDIMCVKKILDKVGINYWL AHKAL
2825.4099	101-124	2	MSO: 109 2841.4048	DDDLTDDIMCVKKILDKVGI NYWL
2460.0950	51-72	1		HTSGYDTQAIVQNNDSTEYG LF
2367.2450	105-124	1	MSO: 109 2383.2399	TDDIMCVKKILDKVGINYWL
2313.0265	51-71	0		HTSGYDTQAIVQNNDSTEYG L
2188.1583	116-134	2		DKVGINYWLAHKALCSEKL
2100.0470	32-50	2		KDLKGYGGVSLPEWVCTTF
1849.9285	100-115	2	MSO: 109 1865.9234	LDDDLTDDIMCVKKIL
1743.8410	35-50	1		KGYGGVSLPEWVCTTF
1736.8445	101-115	1	MSO: 109 1752.8394	DDDLTDDIMCVKKIL
1641.8417	125-138	2		AHKALCSEKLDQWL
1627.8954	116-129	1		DKVGINYWLAHKAL
1594.7603	130-142	2		CSEKLDQWLCEKL
1534.8587	29-42	2		RELKDLKGYGGVSL
1494.7621	20-31	2		EQLTKCEVFREL
1480.7828	23-34	2		TKCEVFRELKDL
1278.6796	105-115	0	MSO: 109 1294.6745	TDDIMCVKKIL
1136.6310	32-42	1		KDLKGYGGVSL
1124.5768	23-31	1		TKCEVFREL
1121.5295	130-138	1		CSEKLDQWL
1107.5833	116-124	0		DKVGINYWL

1099.5928	125-134	1		AHKALCSEKL
1096.5343	20-28	1		EQLTKCEVF
1034.4975	135-142	1		DQWLCEKL
982.4339	43-50	0		PEWVCTTF
780.4250	35-42	0		KGYGGVSL
773.4515	29-34	1		RELKDL
726.3491	23-28	0		TKCEVF
590.2668	100-104	1		LDDDL
579.2807	130-134	0		CSEKL
561.2667	135-138	0		DQWL
539.3300	125-129	0		AHKAL
492.2486	139-142	0		CEKL
477.1827	101-104	0		DDDL
417.2456	29-31	0		REL
389.2031	20-22	0		EQL
375.2238	32-34	0		KDL
166.0862	72-72	0		F
132.1019	100-100	0		L

Enzyme: Pepsin (pH 1.3)

Protein: Beta-lactoglobulin precursor (Beta-LG) (Allergen Bos d 5)

Maximum number of missed cleavages (MC): 2

All cysteines are in reduced form. Methionine have been oxidized to form methionine sulfoxide (MSO).

Using monoisotopic masses of the occurring amino acid residues and giving peptide masses as $[M+H]^+$.

Mass	Position	#MC	Artif.modification(s)	Peptide sequence
3997.1942	75-109	2		QKWENGECAQKKIIAEKTKI PAVFKIDALNENKVL
3412.9024	74-103	2		LQKWENGECAQKKIIAEKTK IPAVFKIDAL
3299.8183	75-103	1		QKWENGECAQKKIIAEKTKI PAVFKIDAL
3227.7860	71-98	2		EILLQKWENGECAQKKIIAE KTKIPAVF
3211.6741	18-47	2	MSO: 23, 40 3243.6639	IVTQTMKGLDIQKVAGTWYS LAMAASDISL
3080.3315	122-149	2	MSO: 123 3096.3264	CMENSAEPEQSLACQCLVRT PEVDDEAL
2872.5753	74-98	1		LQKWENGECAQKKIIAEKTK IPAVF
2759.4912	75-98	0		QKWENGECAQKKIIAEKTKI PAVF
2465.3472	17-38	2	MSO: 23 2481.3421	LIVTQTMKGLDIQKVAGTWY SL
2427.2401	49-70	2		DAQSAPLRVYVEELKPTPEG DL
2353.2108	27-48	2	MSO: 40 2369.2057	DIQKVAGTWYSLAMAASDIS LL
2352.2631	18-38	1	MSO: 23 2368.2580	IVTQTMKGLDIQKVAGTWYS L
2240.1267	27-47	1	MSO: 40 2256.1216	DIQKVAGTWYSLAMAASDIS L
2166.0205	134-152	2		ACQCLVRTPEVDDEALEKF
2100.1222	56-73	2		RVYVEELKPTPEGDLEIL
2075.0655	139-156	2		VRTPEVDDEALEKFDKAL
2002.8013	121-138	2	MSO: 123 2018.7962	FCMENSAEPEQSLACQCL
1955.0484	104-119	2		NENKVLVLDTDYKKYL
1855.7329	122-138	1	MSO: 123 1871.7278	CMENSAEPEQSLACQCL
1761.8146	134-149	1		ACQCLVRTPEVDDEAL
1744.9115	56-70	1		RVYVEELKPTPEGDL
1702.9010	48-62	2		LDAQSAPLRVYVEEL
1673.8414	39-55	2	MSO: 40 1689.8363	AMAASDISLLDAQSAPL
1647.8224	139-152	1		VRTPEVDDEALEKF
1597.6872	120-133	2	MSO: 123 1613.6822	LFCMENSAEPEQSL
1589.8169	49-62	1		DAQSAPLRVYVEEL
1553.8257	160-172	2	MSO: 161 1569.8206	PMHIRLSFNPTQL
1545.7002	166-178	2		SFNPTQLEEQCHI

1505.8984	153-165	2	MSO: 161 1521.8933	DKALKALPMHIRL
1484.6032	121-133	1	MSO: 123 1500.5981	FCMENSAEPEQSL
1468.8733	99-111	2		KIDALNENKVLVL
1380.7158	27-38	0		DIQKVAGTWYSL
1370.7566	110-120	2		VLDTDYKKYLL
1337.5348	122-133	0	MSO: 123 1353.5297	CMENSAEPEQSL
1324.7358	63-74	2		KPTPEGDLEILL
1312.7558	157-167	2	MSO: 161 1328.7507	KALPMHIRLSF
1311.5998	168-178	1		NPTQLEEQCHI
1305.6725	112-121	2		DTDYKKYLLF
1257.6725	110-119	1		VLDTDYKKYL
1256.7208	99-109	1		KIDALNENKVL
1243.6164	139-149	0		VRTPEVDDEAL
1211.6517	63-73	1		KPTPEGDLEIL
1162.6830	150-159	2		EKFDKALKAL
1158.6041	112-120	1		DTDYKKYLL
1103.6493	17-26	1	MSO: 23 1119.6442	LIVTQTMKGL
1078.6553	157-165	1	MSO: 161 1094.6503	KALPMHIRL
1045.5200	112-119	0		DTDYKKYL
1000.5397	160-167	1	MSO: 161 1016.5346	PMHIRLSF
991.5128	39-48	1	MSO: 40 1007.5077	AMAASDISLL
990.5652	18-26	0	MSO: 23 1006.5601	IVTQTMKGL
928.5462	104-111	1		NENKVLVL
907.4883	56-62	0		RVYVEEL
878.4288	39-47	0	MSO: 40 894.4237	AMAASDISL
856.4410	63-70	0		KPTPEGDL
850.4669	150-156	1		EKFDKAL
814.4305	48-55	1		LDAQSAPL
806.4043	166-172	1		SFNPTQL
766.4392	160-165	0	MSO: 161 782.4341	PMHIRL
758.4770	153-159	1		DKALKAL
758.3137	173-178	0		EEQCHI
716.3937	104-109	0		NENKVL
701.3464	49-55	0		DAQSAPL
572.3038	168-172	0		NPTQL
559.3450	99-103	0		KIDAL
537.2160	134-138	0		ACQCL
487.3126	71-74	1		EILL
446.2609	153-156	0		DKAL
423.2238	150-152	0		EKF
374.2285	71-73	0		EIL

331.2340	157-159	0		KAL
279.1703	120-121	1		LF
253.1183	166-167	0		SF
231.1703	110-111	0		VL
166.0862	121-121	0		F
132.1019	17-17	0		L
132.1019	48-48	0		L
132.1019	74-74	0		L
132.1019	120-120	0		L

Enzyme: Pepsin (pH > 2).

Protein: Alpha-lactalbumin (Lactose synthase B protein) (Allergen Bos d 4)

Maximum number of missed cleavages (MC): 2

All cysteines are in reduced form. Methionine have been oxidized to form methionine sulfoxide (MSO).

Using monoisotopic masses of the occurring amino acid residues and giving peptide masses as $[M+H]^+$.

Mass	Position	#MC	Artif.modification(s)	Peptide sequence
3036.3760	74-99	2		INNKIWCKDDQNPHSSNICN ISCDKF
2526.2465	101-122	2	MSO: 109 2542.2415	DDDLTDDIMCVKKILDKVGI NY
2381.0319	80-100	2		CKDDQNPHSSNICNISCDKF L
2267.9478	80-99	1		CKDDQNPHSSNICNISCDKF
2254.1610	105-123	2	MSO: 109 2270.1559	TDDIMCVKKILDKVGINYW
2249.9801	85-104	2		NPHSSNICNISCDKFLDDDL
2068.0817	105-122	1	MSO: 109 2084.0766	TDDIMCVKKILDKVGINY
1849.9285	100-115	2	MSO: 109 1865.9234	LDDDLTDDIMCVKKIL
1791.8152	85-100	1		NPHSSNICNISCDKFL
1736.8445	101-115	1	MSO: 109 1752.8394	DDDLTDDIMCVKKIL
1678.7312	85-99	0		NPHSSNICNISCDKF
1504.7213	73-84	2		QINNKIWCKDDQ
1459.6158	46-58	2		VCTTFHTSGYDTQ
1376.6627	74-84	1		INNKIWCKDDQ
1301.5619	45-55	2		WVCTTFHTSGY
1278.6796	105-115	0	MSO: 109 1294.6745	TDDIMCVKKIL
1182.5273	60-69	2		IVQNNDSTEY
1136.6310	32-42	2		KDLKGYGGVSL
1115.4826	46-55	1		VCTTFHTSGY
1107.5833	116-124	2		DKVGINYWL
1090.5011	59-68	2		AIVQNNDSTE
1062.5731	72-79	2		FQINNKIW
1019.4640	60-68	1		IVQNNDSTE
1012.4218	63-71	2		NNDSTEYGL
1011.4928	23-30	2		TKCEVFRE
1006.5203	35-44	2		KGYGGVSLPE
994.4992	116-123	1		DKVGINYW
982.4339	43-50	2		PEWVCTTF
979.4115	51-59	2		HTSGYDTQA
915.5047	73-79	1		QINNKIW
908.3744	51-58	1		HTSGYDTQ

844.4199	38-45	2		GGVSLPEW
842.3163	63-69	1		NNDSTEY
839.4331	22-28	2		LTKCEVF
836.4876	31-37	2		LKDLKGY
822.3662	130-136	2		CSEKLDQ
808.4199	116-122	0		DKVGINY
787.4461	74-79	0		INNKIW
787.3767	126-132	2		HKALCSE
780.4250	35-42	1		KGYGGVSL
774.3992	56-62	2		DTQAIVQ
773.4515	29-34	2		RELKDL
756.3385	45-50	1		WVCTTF
726.3491	23-28	1		TKCEVF
723.4035	32-37	1		KDLKGY
721.3549	21-26	2		QLTKCE
692.3647	129-134	2		LCSEKL
689.3617	133-137	2		KLDQW
679.2529	63-68	0		NNDSTE
663.3824	27-31	2		VFREL
658.3406	38-44	1		GGVSLPE
608.2344	80-84	0		CKDDQ
605.3327	138-142	2		LCEKL
593.2963	22-26	1		LTKCE
590.2668	100-104	1		LDDDL
579.2807	130-134	1		CSEKL
570.2592	46-50	0		VCTTF
564.2412	51-55	0		HTSGY
561.2667	135-138	2		DQWL
550.2983	27-30	1		VFRE
550.2330	137-140	2		WLCE
539.3300	124-128	2		LAHKA
539.3300	125-129	2		AHKAL
503.2824	133-136	1		KLDQ
499.2551	69-72	2		YGLF
492.2486	139-142	1		CEKL
488.3078	31-34	1		LKDL
480.2122	23-26	0		TKCE
477.1827	101-104	0		DDDL
468.2929	126-129	1		HKAL
464.2503	70-73	2		GLFQ
451.1857	129-132	1		LCSE
448.1827	135-137	1		DQW
434.1881	56-59	1		DTQA
432.2452	38-42	0		GGVSL

431.1925	43-45	1		PEW
430.2660	59-62	1		AIVQ
426.2459	125-128	1		AHKA
417.2456	29-31	1		REL
389.2183	123-125	2		WLA
389.2031	20-22	2		EQL
375.2238	32-34	0		KDL
367.1976	35-37	0		KGY
364.1537	138-140	1		LCE
363.1510	56-58	0		DTQ
359.2289	60-62	0		IVQ
355.2088	126-128	0		HKA
352.1867	69-71	1		YGL
338.1016	130-132	0		CSE
336.1918	70-72	1		GLF
318.1812	123-124	1		WL
318.1812	137-138	1		WL
304.1615	29-30	0		RE
294.1448	72-73	1		FQ
276.1190	20-21	1		EQ
265.1546	27-28	0		VF
262.1033	135-136	0		DQ
260.1968	133-134	0		KL
260.1968	141-142	0		KL
260.1605	21-22	1		QL
251.0696	139-140	0		CE
245.1132	43-44	0		PE
205.0971	45-45	0		W
205.0971	123-123	0		W
205.0971	137-137	0		W
203.1390	124-125	1		LA
189.1233	70-71	0		GL
182.0812	69-69	0		Y
166.0862	72-72	0		F
148.0604	20-20	0		E
147.0764	21-21	0		Q
147.0764	73-73	0		Q
132.1019	22-22	0		L
132.1019	31-31	0		L
132.1019	100-100	0		L
132.1019	124-124	0		L
132.1019	129-129	0		L
132.1019	138-138	0		L
90.0549	59-59	0		A

90.0549	125-125	0		A

Enzyme: Pepsin (pH > 2).

Protein: Beta-lactoglobulin precursor (Beta-LG) (Allergen Bos d 5)

Maximum number of missed cleavages (MC): 2

All cysteines are in reduced form. Methionine have been oxidized to form methionine sulfoxide (MSO).

Using monoisotopic masses of the occurring amino acid residues and giving peptide masses as $[M+H]^+$.

Mass	Position	#MC	Artif.modification(s)	Peptide sequence
1440.7416	160-171	2	MSO: 161 1456.7365	PMHIRLSFNPTQ
1346.7348	18-29	2	MSO: 23 1362.7297	IVTQTMKGLDIQ
1339.8671	85-96	2		KKIIAEKTKIPA
1330.8092	91-102	2		KTKIPAVFKIDA
1275.5885	137-147	2		CLVRTPEVDDE
1203.6765	22-32	2	MSO: 23 1219.6714	TMKGLDIQKVA
1179.6255	106-115	2		NKVLVLDTDY
1144.5884	110-118	2		VLDTDYKKY
1130.5324	139-148	2		VRTPEVDDEA
1113.6237	159-167	2	MSO: 161 1129.6186	LPMHIRLSF
1103.6493	17-26	2	MSO: 23 1119.6442	LIVTQTMKGL
1078.6553	157-165	2	MSO: 161 1094.6503	KALPMHIRL
1059.4953	139-147	1		VRTPEVDDE
1048.4914	135-143	2		CQCLVRTPE
1045.5200	112-119	2		DTDYKKYL
1032.6088	90-98	2		EKTKIPAVF
1017.5363	27-35	2		DIQKVAGTW
1000.5397	160-167	1	MSO: 161 1016.5346	PMHIRLSF
990.5652	18-26	1	MSO: 23 1006.5601	IVTQTMKGL
985.4836	63-71	2		KPTPEGDLE
969.5251	62-70	2		LKPTPEGDL
932.4360	112-118	1		DTDYKKY
928.5462	104-111	2		NENKVLVL
905.4761	22-29	1	MSO: 23 921.4710	TMKGLDIQ
903.5662	91-98	1		KTKIPAVF
879.5233	159-165	1	MSO: 161 895.5182	LPMHIRL
875.4985	54-60	2		PLRVYVE
874.5179	82-89	2		CAQKKIIA
856.4410	63-70	1		KPTPEGDL

833.4251	43-50	2		SDISLLDA
829.5141	84-90	2		QKKIIAE
829.4778	103-109	2		LNENKVL
824.4301	30-36	2		KVAGTWY
817.4236	137-143	1		CLVRTPE
813.4352	61-67	2		ELKPTPE
807.3917	40-47	2	MSO: 40 823.3866	MAASDISL
806.4043	166-172	2		SFNPTQL
805.4818	97-103	2		VFKIDAL
805.4566	52-58	2		SAPLRVY
802.4305	99-105	2		KIDALNE
801.2906	121-127	2	MSO: 123 817.2855	FCMENSA
794.4043	56-61	2		RVYVEE
786.4719	90-96	1		EKTKIPA
783.2648	122-128	2	MSO: 123 799.2597	CMENSAE
766.4392	160-165	0	MSO: 161 782.4341	PMHIRL
762.3417	76-81	2		KWENGE
737.3828	150-155	2		EKFDKA
726.3457	33-38	2		GTWYSL
725.3352	110-115	1		VLDTDY
721.4243	151-156	2		KFDKAL
718.3981	42-48	2		ASDISLL
716.3937	104-109	1		NENKVL
701.4556	85-90	1		KKIIAE
701.3464	168-173	2		NPTQLE
700.4716	84-89	1		QKKIIA
693.3202	166-171	1		SFNPTQ
692.3977	97-102	1		VFKIDA
685.4607	106-111	1		NKVLVL
684.3926	62-67	1		LKPTPE
673.3879	27-32	1		DIQKVA
665.3617	56-60	1		RVYVE
664.4392	116-120	2		KKYLL
661.3668	30-35	1		KVAGTW
661.3039	144-149	2		VDDEAL
659.3610	68-73	2		GDLEIL
657.4294	91-96	0		KTKIPA
654.2222	122-127	1	MSO: 123 670.2171	CMENSA
647.3875	54-58	1		PLRVY
647.3610	43-48	1		SDISLL
646.2678	125-130	2		NSAEPE
645.3930	153-158	2		DKALKA

642.2626	120-124	2	MSO: 123 658.2575	LFCME
629.2712	174-178	2		EQCHI
622.2137	78-83	2		ENGECA
621.2297	79-84	2		NGECAQ
608.3402	151-155	1		KFDKA
605.3141	42-47	1		ASDISL
601.3304	139-143	0		VRTPE
590.2933	75-78	2		QKWE
574.3347	74-77	2		LQKW
573.3606	17-21	1		LIVTQ
573.2878	129-133	2		PEQSL
572.4130	85-89	0		KKIIA
572.3038	168-172	1		NPTQL
571.3086	63-67	0		KPTPE
559.3450	99-103	1		KIDAL
551.3551	116-119	1		KKYL
549.3065	22-26	0	MSO: 23 565.3014	TMKGL
548.2198	144-148	1		VDDEA
537.2160	134-138	2		ACQCL
536.3078	149-152	2		LEKF
534.2769	43-47	0		SDISL
529.1785	121-124	1	MSO: 123 545.1734	FCME
526.2296	33-36	1		GTWY
521.2388	132-136	2		SLACQ
515.2824	51-55	2		QSAPL
513.1827	112-115	0		DTDY
502.2143	128-131	2		EPEQ
500.2286	175-178	1		QCHI
493.1711	79-83	1		NGECA
492.2486	37-41	2	MSO: 40 508.2435	SLAMA
491.2096	49-53	2		DAQSA
489.2555	59-62	2		VEEL
487.3126	71-74	2		EILL
486.3286	72-75	2		ILLQ
477.1827	144-147	0		VDDE
473.3082	106-109	0		NKVL
466.1788	135-138	1		CQCL
462.2347	76-78	1		KWE
461.2507	75-77	1		QKW
460.2766	18-21	0		IVTQ
459.2198	168-171	0		NPTQ
453.2344	36-39	2		YSLA
448.1674	78-81	1		ENGE

446.2609	99-102	0		KIDA
446.2609	153-156	1		DKAL
446.2245	48-51	2		LDAQ
444.3180	156-159	2		LKAL
438.2711	116-118	0		KKY
437.2507	56-58	0		RVY
433.1929	68-71	1		GDLE
423.2238	150-152	1		EKF
420.1725	125-128	1		NSAE
418.2296	131-134	2		QSLA
405.1616	173-175	2		EEQ
392.2544	119-121	2		LLF
390.1871	172-174	2		LEE
387.2238	52-55	1		SAPL
382.1972	36-38	1		YSL
382.1101	122-124	0	MSO: 123 398.1050	CME
376.1714	59-61	1		VEE
375.1874	27-29	0		DIQ
375.1874	103-105	1		LNE
374.2285	71-73	1		EIL
374.1558	128-130	1		EPE
373.1718	129-131	1		PEQ
372.1700	176-178	0		CHI
363.1696	39-42	2	MSO: 40 379.1646	AMAA
363.1663	33-35	0		GTW
358.2700	72-74	1		ILL
347.1925	131-133	1		QSL
333.1921	76-77	0		KW
333.1768	153-155	0		DKA
333.1405	49-51	1		DAQ
332.1816	148-150	2		ALE
331.2340	156-158	1		LKA
331.2340	157-159	1		KAL
321.1227	82-84	1		CAQ
321.1227	134-136	1		ACQ
319.1248	79-81	0		NGE
318.1659	48-50	1		LDA
317.2183	30-32	0		KVA
305.1455	51-53	1		QSA
304.1503	68-70	0		GDL
294.1812	151-152	0		KF
292.1325	39-41	1	MSO: 40 308.1274	AMA
292.1325	40-42	1	MSO: 40 308.1274	MAA

291.1299	125-127	0		NSA
290.1710	37-39	1		SLA
290.1710	132-134	1		SLA
279.1703	120-121	1		LF
277.1030	173-174	1		EE
276.1190	174-175	1		EQ
265.1546	97-98	0		VF
262.1033	104-105	0		NE
261.1445	61-62	1		EL
261.1445	149-150	1		LE
261.1445	172-173	1		LE
260.1605	74-75	1		LQ
253.1183	166-167	0		SF
250.0856	135-136	0		CQ
247.1288	59-60	0		VE
245.1859	72-73	0		IL
245.1859	119-120	1		LL
245.1132	129-130	0		PE
235.1111	137-138	0		CL
231.1703	110-111	0		VL
229.1546	54-55	0		PL
221.0954	40-41	0	MSO: 40 237.0903	MA
219.1339	37-38	0		SL
219.1339	132-133	0		SL
218.1499	157-158	0		KA
205.0819	49-50	0		DA
203.1390	148-149	1		AL
193.0641	82-83	0		CA
182.0812	36-36	0		Y
177.0870	52-53	0		SA
166.0862	121-121	0		F
148.0604	61-61	0		E
148.0604	71-71	0		E
148.0604	78-78	0		E
148.0604	90-90	0		E
148.0604	128-128	0		E
148.0604	150-150	0		E
148.0604	173-173	0		E
148.0604	174-174	0		E
147.0764	51-51	0		Q
147.0764	75-75	0		Q
147.0764	84-84	0		Q
147.0764	131-131	0		Q
147.0764	175-175	0		Q

132.1019	17-17	0		L
132.1019	48-48	0		L
132.1019	62-62	0		L
132.1019	74-74	0		L
132.1019	103-103	0		L
132.1019	119-119	0		L
132.1019	120-120	0		L
132.1019	149-149	0		L
132.1019	156-156	0		L
132.1019	159-159	0		L
132.1019	172-172	0		L
90.0549	39-39	0		A
90.0549	42-42	0		A
90.0549	134-134	0		A
90.0549	148-148	0		A

Enzyme: Chymotrypsin (C-term to F/Y/W, not before P)

Protein: Alpha-lactalbumin (Lactose synthase B protein) (Allergen Bos d 4)

Maximum number of missed cleavages (MC): 2

All cysteines are in reduced form. Methionine have been oxidized to form methionine sulfoxide (MSO).

Using monoisotopic masses of the occurring amino acid residues and giving peptide masses as $[M+H]^+$.

Mass	Position	#MC	Artif.modification(s)	Peptide sequence
5784.7474	73-122	2	MSO: 109 5800.7423	QINNKIWCKDDQNPHSSNIC NISCDKFLDDDLTDDIMCVK KILDKVGINY
5074.3399	80-123	2	MSO: 109 5090.3348	CKDDQNPHSSNICNISCDKF LDDDLTDDIMCVKKILDKVG INYW
4888.2606	80-122	1	MSO: 109 4904.2555	CKDDQNPHSSNICNISCDKF LDDDLTDDIMCVKKILDKVG INY
4448.2338	100-137	2	MSO: 109 4464.2287	LDDDLTDDIMCVKKILDKVG INYWLAHKALCSEKLDQW
3481.6086	70-99	2		GLFQINNKIWCKDDQNPHSS NICNISCDKF
3164.4346	73-99	1		QINNKIWCKDDQNPHSSNIC NISCDKF
3024.5498	20-45	2		EQLTKCEVFRELKDLKGYGG VSLPEW
2825.4099	100-123	1	MSO: 109 2841.4048	LDDDLTDDIMCVKKILDKVG INYW
2811.3584	56-79	2		DTQAIVQNNDSTEYGLFQIN NKIW
2694.1624	46-69	2		VCTTFHTSGYDTQAIVQNND STEY
2639.3306	100-122	0	MSO: 109 2655.3255	LDDDLTDDIMCVKKILDKVG INY
2498.2747	29-50	2		RELKDLKGYGGVSLPEWVCT TF
2460.0950	51-72	2		HTSGYDTQAIVQNNDSTEYG LF
2414.2359	123-142	2		WLAHKALCSEKLDQWLCEKL
2267.9478	80-99	0		CKDDQNPHSSNICNISCDKF
2228.1566	124-142	1		LAHKALCSEKLDQWLCEKL
2199.1478	20-37	1		EQLTKCEVFRELKDLKGY
2142.9210	51-69	1		HTSGYDTQAIVQNNDSTEY
1947.0334	29-45	1		RELKDLKGYGGVSLPEW
1940.8847	38-55	2		GGVSLPEWVCTTFHTSGY
1914.8715	56-72	1		DTQAIVQNNDSTEYGLF
1827.9210	123-137	1		WLAHKALCSEKLDQW
1641.8417	124-137	0		LAHKALCSEKLDQW
1597.6976	56-69	0		DTQAIVQNNDSTEY
1395.6613	38-50	1		GGVSLPEWVCTTF
1232.6786	70-79	1		GLFQINNKIW
1121.6313	29-37	0		RELKDLKGY
1115.4826	46-55	1		VCTTFHTSGY
1096.5343	20-28	0		EQLTKCEVF
915.5047	73-79	0		QINNKIW
844.4199	38-45	0		GGVSLPEW

605.3327	138-142	0		LCEKL
570.2592	46-50	0		VCTTF
564.2412	51-55	0		HTSGY
336.1918	70-72	0		GLF
205.0971	123-123	0		W

Enzyme: Chymotrypsin (C-term to F/Y/W, not before P)

Protein: Beta-lactoglobulin precursor (Beta-LG) (Allergen Bos d 5)

Maximum number of missed cleavages (MC): 2

All cysteines are in reduced form. Methionine have been oxidized to form methionine sulfoxide (MSO).

Using monoisotopic masses of the occurring amino acid residues and giving peptide masses as $[M+H]^+$.

Mass	Position	#MC	Artif.modification(s)	Peptide sequence
6808.6194	37-98	2	MSO: 40 6824.6143	SLAMAASDISLLDAQSAPLR VYVEELKPTPEGDLEILLQK WENGECAQKKIIAEKTKIPA VF
6498.1004	122-178	2	MSO: 123, 161 6530.0903	CMENSAEPEQSLACQCLVRT PEVDDEALEKFDKALKALPM HIRLSFNPTQLEEQCHI
6479.4672	59-115	2		VEELKPTPEGDLEILLQKWE NGECAQKKIIAEKTKIPAVF KIDALNENKVLVLDTDY
5578.7550	119-167	2	MSO: 123, 161 5610.7449	LLFCMENSAEPEQSLACQCL VRTPEVDDEALEKFDKALKA LPMHIRLSF
5205.5185	122-167	1	MSO: 123, 161 5237.5083	CMENSAEPEQSLACQCLVRT PEVDDEALEKFDKALKALPM HIRLSF
4680.5320	78-118	2		ENGECAQKKIIAEKTKIPAV FKIDALNENKVLVLDTDYKK Y
4673.4422	36-77	2	MSO: 40 4689.4371	YSLAMAASDISLLDAQSAPL RVYVEELKPTPEGDLEILLQ KW
4538.4036	17-58	2	MSO: 23, 40 4570.3935	LIVTQTMKGLDIQKVAGTWY SLAMAASDISLLDAQSAPLR VY
4535.4468	59-98	1		VEELKPTPEGDLEILLQKWE NGECAQKKIIAEKTKIPAVF
4510.3788	37-77	1	MSO: 40 4526.3737	SLAMAASDISLLDAQSAPLR VYVEELKPTPEGDLEILLQK W
4277.0272	116-152	2	MSO: 123 4293.0222	KKYLLFCMENSAEPEQSLAC QCLVRTPEVDDEALEKF
4261.2787	78-115	1		ENGECAQKKIIAEKTKIPAV FKIDALNENKVLVLDTDY
3857.7740	119-152	1	MSO: 123 3873.7689	LLFCMENSAEPEQSLACQCL VRTPEVDDEALEKF
3484.5375	122-152	0	MSO: 123 3500.5324	CMENSAEPEQSLACQCLVRT PEVDDEALEKF
3032.5808	153-178	1	MSO: 161 3048.5757	DKALKALPMHIRLSFNPTQL EEQCHI
2755.5280	99-121	2		KIDALNENKVLVLDTDYKKY LLF
2455.2537	36-58	1	MSO: 40 2471.2486	YSLAMAASDISLLDAQSAPL RVY
2382.2914	99-118	1		KIDALNENKVLVLDTDYKKY
2317.2584	78-98	0		ENGECAQKKIIAEKTKIPAV F
2292.1904	37-58	0	MSO: 40 2308.1853	SLAMAASDISLLDAQSAPLR VY
2265.2311	17-36	1	MSO: 23 2281.2260	LIVTQTMKGLDIQKVAGTWY
2237.2063	59-77	0		VEELKPTPEGDLEILLQKW
2102.1678	17-35	0	MSO: 23 2118.1627	LIVTQTMKGLDIQKVAGTW
1963.0382	99-115	0		KIDALNENKVLVLDTDY
1739.9989	153-167	0	MSO: 161 1755.9938	DKALKALPMHIRLSF
1311.5998	168-178	0		NPTQLEEQCHI
811.5076	116-121	1		KKYLLF

438.2711	116-118	0		KKY
392.2544	119-121	0		LLF
182.0812	36-36	0		Y

Appendix 4: Ranking test

Name : **Surname :**
Smoker (Y/N) : **Date :**

Characteristic studied : **Preference of appearance and aroma**

Part A : Preference of the appearance

Instructions:

1. Receive the sample tray with the two rows of samples and note each sample code below according to its position on the tray.
2. Starting from the first row, look at the samples from left to right and note the degree of preference to their appearance
   Write "**1**" in the box for the sample's appearance you **prefer the least**
   Write "2" for the next , "3" for the next, and "**4**" for the one you **prefer the most**
   You may find it helpful to first arrange the samples in a provisional order, and then resolve the positions of adjacent samples by more careful observation.
3. If two samples appear the same, please make a "best guess" as to their rank order.
4. Repeat steps 2-3 for the second row of samples.
5. In the end of the test please put the samples back to their original serving order. Thank you!

1st row: Sample Code _____ _____ _____ _____

Rank ☐ ☐ ☐ ☐

2nd row: Sample Code _____ _____ _____ _____

Rank ☐ ☐ ☐ ☐

Comments : _____

_____

_____

_____

_____

_____

End of part A ! New samples will be served for part B !

<u>Part B :</u> Preference of the aroma

<u>Instructions:</u>

1. Receive the sample tray and note each sample code below according to its position on the tray.
2. Smell the samples from left to right and note the
   <u>degree of preference for the aroma</u>
   Wait at least 15 seconds between samples and cleanse your nose as needed by smelling your wrist .
3. Write "**1**" in the box of the sample's aroma you **<u>prefer the least</u>**
   Write "2" for the next , "3" for the next, and "**4**" for the one you **<u>prefer the most</u>**
   You may find it helpful to first arrange the samples in a provisional order, and then resolve the positions of adjacent samples by more careful smelling.
4. If two samples appear the same, make a "best guess" as to their rank order.
5. In the end of the test please put the samples back to their original serving order.
6. Repeat steps 1-5 for the next row of samples which will be served after 5 minutes.
   Thank you!

1st row: Sample Code _____ _____ _____ _____

Rank ☐ ☐ ☐ ☐

2nd row: Sample Code _____ _____ _____ _____

Rank ☐ ☐ ☐ ☐

<u>Comments :</u> _____

_____

_____

_____

_____

_____

Thank you for your participation!

Summary

SUMMARY

This PhD thesis contributes to a better understanding of the flavor chemistry during the Maillard reaction by, (1) developing food related model systems, (2) monitoring their production of volatiles, more specifically the generation of pyrazines, (3) reporting and investigating their formation mechanism, (4) elucidating the role of various factors and components of the Maillard model systems for the generation of pyrazines, and (5) applying the information obtained through the model systems into real food products.

Chapter 1 contains a literature overview on peptides, their generalities and abundance in food products. The second part is dedicated to the definition of the Maillard reaction, the factors that have an influence on it, its importance as a generator of flavor compounds and a review of the available studies in model systems containing proteins, peptides and amino acids.

Chapter 2 initially describes the necessity of developing Maillard model systems which would contain proteins and peptides, as their overall influence on the Maillard reaction was less studied so far. Further, it describes the hydrolysis of the whey protein as a source of peptides and free amino acids. The second part studies the thermal reaction conditions, which were selected to imitate commercial sterilization and roasting, with the objective of finding the influence of the type of reactant and ratios of the reactant by using several model systems. The third part of this chapter contains the elucidation of the role of tryptic protein hydrolysis products in the formation of pyrazines, for which the impact of each component on the model system is evaluated. The final part of the chapter studies the impact of a_w on pyrazine generation using the hydrolyzed whey protein model systems. The overall results of this chapter show that the generation of pyrazines in Maillard model systems was enhanced at high temperatures (> 120 ^oC) and at low water activities (0.33). However, the main finding was the fact that peptides contained in the studied hydrolysate contributed to a greater extent towards the formation of pyrazines compared with free amino acids, and that, therefore, their role as a flavor precursor has been underestimated.

Chapter 3 describes the fractionation and characterization of whey protein hydrolysate obtained upon digestion with trypsin. The hydrolysate was divided in six fractions obtained via preparative gel permeation chromatography and further characterized for their total amino acid content and their capacity to generate pyrazines. It was found that all the fractions were able to generate pyrazines in the Maillard model systems. However, these pyrazines were produced in higher amount in fractions of low molecular weight. The fractions were then further analyzed via MALDI-TOF-MS and MALDI-TOF-tandem MS with the intention to determine the masses of the peptides and, if possible, to identify them. The identification with MALDI-TOF-tandem MS was only possible for three peptides due to the small quantities present in the sample. However, several other peptides were partially

identified using a validated database. Since the sequences and the *N-t*erminal amino acid of the identified peptides were of various kind, it was concluded that they did not influence the generation of pyrazines.

Chapter 4 describes the generation of pyrazines in model systems containing hydrolyzed whey protein in dry heating conditions. Based on the findings described in **Chapter 2**, five enzymatic hydrolyses were performed on the whey protein to obtain different peptidic profiles. The hydrolysates were characterized by gel permeation chromatography (GPLC) and high performance liquid chromatography (HPLC). The impact of each hydrolysate and the ratio of the reactants was measured by headspace solid-phase microextraction coupled with gas chromatography/mass spectrometry (HS-SPME-GC/MS). Both factors showed a dramatic influence on the generation of pyrazines. Also the presence of oligopeptides had an enhancing role in the generation of pyrazines while in contrast the free amino acids contributed to a minor extent. Additional experiments were performed by using α-dicarbonyl compounds in the model experiments in order to further elucidate the different mechanisms of pyrazine formation. As a result, some pyrazines were found to be amino acid specific while others were produced more abundantly in peptide containing systems. Beside this, the different formation pathways of pyrazines are also discussed.

After confirming that peptides are remarkable precursors for the formation of alkylpyrazines, two different whey protein hydrolysates obtained upon enzymatic digestion with trypsin and proteinase from *Aspergillus melleus* (**Chapter 3** and **Chapter 4**) were added as part of the ingredients of two common baked products, bread and cookies (**Chapter 5**). The generation of 2,5-dimethylpyrazine was quantified by headspace extraction coupled with gas chromatography/mass spectrometry (HS-GC/MS) and isotope dilution. Moreover the samples were evaluated for aroma and overall appearance by a sensory panel.

The use of both hydrolysates had a remarkable impact on the generation of 2,5-dimethylpyrazine compared to the control samples. Further, the two baked food products generated different quantities of 2,5-dimethylpyrazine, exposing the necessity of applying the existing model systems into a real food matrix. Moreover, the use of both hydrolysates generated different aromas which were clearly differentiated by the panelists. These results showed that the peptides were more effective as precursors for the formation of 2,5-dimethylpyrazine in both food products. Hydrolysates that contained a high amount of free amino acids gave rise to formation of higher amounts of 2,5-dimethylpyrazine and other Maillard characteristic volatile compounds which were however found to be sensorially undesirable.

SAMENVATTING

Deze doctoraatsthesis draagt bij tot het verbeteren van het inzicht in reacties die aanleiding geven tot de vorming van aromacomponenten tijdens de Maillardreactie via (1) de ontwikkeling van voedselgerelateerde modelsystemen, (2) het opvolgen van de vorming van vluchtige componenten, meer specifiek pyrazines, (3) het rapporteren en onderzoeken van hun vormingsmechanismen, (4) het ophelderen van de rol van verschillende factoren en componenten van het Maillard modelsysteem in de vorming van pyrazines en (5) het toepassen van de gegevens bekomen via de modelsystemen in echte voedingsproducten.

Hoofdstuk 1 omvat een literatuuronderzoek omtrent peptiden, hun algemene kenmerken en hun voorkomen in voedingsproducten. Het tweede deel wordt gewijd aan de definitie van de Maillardreactie, de factoren die van invloed zijn en het belang van de Maillardreactie in de vorming van aromacomponenten. Eveneens wordt een overzicht gegeven van de beschikbare studies in modelsytemen bestaande uit proteïnen, peptiden en aminozuren.

Hoofdstuk 2 beschrijft in eerste instantie de noodzaak tot de ontwikkeling van modelsystemen om de invloed van eiwitten en peptiden op de Maillardreactie te onderzoeken, iets wat in de huidige studies minder aandacht gekregen heeft. Vervolgens wordt de hydrolyse van het weiproteïne als bron van peptiden en vrije aminozuren besproken. In het tweede deel worden thermische reactiecondities gebruikt, specifiek geselecteerd ter imitatie van commerciële sterilisatie en droge verhitting (*roasting),* om de invloed van het type en de ratio's van reactieven te bestuderen. In het derde deel wordt de rol van de trypsinogene hydrolyseproducten bij de vorming van pyrazines opgehelderd, waarbij de impact van elke component op het modelsysteem wordt geëvalueerd. Het laatste deel van dit hoofdstuk behandelt de impact van de wateractiviteit op de vorming van pyrazine vanuit het gehydrolyseerde weiproteïne modelsysteem. De algemene resultaten van dit hoofdstuk tonen aan dat de vorming van pyrazines in de verschillende Maillardmodelsystemen beter was bij hoge temperaturen (>120 °C) en bij lage wateractiviteit (0.33). De grootste ontdekking was echter het feit dat de peptiden in grote mate bijdroegen aan de vorming van pyrazines in vergelijking met de vrije aminozuren en bijgevolg dat hun rol als aromaprecursor onderschat wordt.

Hoofstuk 3 beschrijft de fractionering en de typering van het weiproteïne hydrolysaat verkregen door vertering met trypsine. Het hyrolysaat werd door middel van preparatieve gelpermeatiechromatografie verdeeld in zes delen en verder gekarakteriseerd naar hun totale inhoud van aminozuren en hun capaciteit om pyrazines te genereren. Er werd vastgesteld dat alle fracties in de mogelijkheid waren om in Maillardreactie modelsystemen pyrazines te genereren, waarbij de fracties met een laag moleculair gewicht grotere hoeveelheden pyrazines bleken te bevatten. Met behulp van de MALDI-TOF -MS, werden de fracties geanalyseerd om de massa van de

peptiden te achterhalen en, indien mogelijk, de peptiden ook te identificeren. Door de lage concentratie werden slechts drie peptiden éénduidig geïdentificeerd, maar met behulp van een gevalideerde database werden verschillende andere peptides deels tot geheel gekarakteriseerd. Op basis van de peptidesequenties en het N-terminaal aminozuur kon geconcludeerd worden dat deze factoren de ontwikkeling van pyrazines niet beïnvloedden.

Hoofdstuk 4 handelt over de vorming van pyrazines in modelsystemen op basis van gehydrolyseerde weiproteïnen tijdens droge verhitting.. Gebaseerd op de onderzoeksresultaten uit **hoofdstuk 2** werden 5 enzymatische hydrolyses uitgevoerd om verschillende peptideprofielen van weiproteïnehydrolysaten te bekomen, die gekarakteriseerd werden door middel van gelpermeatiechromatografie (GPLC). Met behulp van de kopruimte vaste-stof microextractie gekoppeld met gaschromatografie/massaspectrometrie (HS-SPME-GC/MS) werd de invloed van elk hydrolysaat en de verhouding van reagentia op de vorming van pyrazines gemeten. Beide factoren toonden een significante invloed op de vorming van pyrazines. Verder had de aanwezigheid van oligopeptiden een gunstige invloed op de vorming van pyrazines, in tegenstelling tot vrije aminozuren die een minder grote invloed vertoonden. Bijkomend onderzoek met gebruik van α-dicarbonylverbindingen in de modelsystemen werd uitgevoerd om de verschillende mechanismen van pyrazinevorming op te helderen. Hieruit kon besloten worden dat sommige pyrazines aminozuurspecifiek zijn, terwijl andere meer algemeen voorkomend zijn in peptidebevattende systemen. Daarnaast worden de verschillende reactiewegen ter vorming van pyrazines besproken.

Na de bevestiging dat peptiden opmerkelijke precursoren zijn voor de vorming van alkylpyrazines, werden twee verschillende hydrolysaten van het weiproteïne, (trypsinogeen hydrolysaat en het hydrolysaat bekomen met proteïnase van *Aspergillus melleus*, zie hoofdstuk 3 en 4), toegevoegd aan twee typische bakkerijproducten, namelijk brood en koekjes (**Hoofdstuk 5**). De vorming van 2,5-dimethylpyrazine werd gekwantificeerd door extractie van de kopruimte gekoppeld met gas chromatografie/massa spectrometrie (HS-GC/MS) en isotoopdilutie. Daarnaast werden de stalen door een sensorisch panel geëvalueerd op aroma en op algemeen voorkomen.

Het gebruik van beide hydrolysaten had een opmerkelijke impact op de vorming van 2,5-dimethylpyrazine in vergelijking met de controle stalen. Verder werden in de twee producten na het bakken verschillende hoeveelheden 2,5-dimethylpyrazine gevormd. Dit toont aan dat het noodzakelijk is om bestaande modellen toe te passen op echte voedingsmatrices. De twee hydrolysaten resulteerden in een verschillend aroma, wat duidelijk werd aangetoond door de panelleden. Deze resultaten tonen aan dat peptides effectieve precursoren zijn voor de vorming van 2,5-dimethylpyrazine in beide voedingsproducten, zelfs wanneer ze in kleine hoeveelheden worden toegevoegd. Wanneer echter hydrolysaten met een hoog gehalte aan vrije aminozuren worden gebruikt, worden naast 2,5-dimethylpyrazine ook vluchtige verbindingen gevormd. Deze

verbindingen, die karakteristiek zijn voor de Maillardreactie, worden als sensorisch ongewenst bevonden.

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Curriculum Vitae

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In preparation:

Scalone, G.L., Ioannidis, A.G., Lamichhane, P., Devlieghere, F., Cadwallader, K., De Kimpe, N. & De Meulenaer, B. Impact of Whey Protein Hydrolysates on the Formation of 2,5-Dimethylpyrazine in Baked Food Products

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Training school: "Food digestion and human health" 24 – 28, March 2014, Budapest, Hungary.

Passive participation:

Influence of free amino acids, oligopeptides and polypeptides on the formation of pyrazines in Maillard model systems. G. Leonardo Scalone, P. Lamichhane, T. Cucu, N. De Kimpe, B.E. De

Meulenaer. 251st American Chemical Society National Meeting, March 13 - 17, 2016. San Diego, California, United States of America.

Supervised dissertation as tutor:

Lamichhane, P. (2015). Effect of amino acids and peptides obtained from the hydrolysis of whey protein isolate by different enzymes on pyrazines formation in Maillard model systems. Master thesis dissertation. Ghent University, Belgium.